OPTIMIZATION OF AMMONIUM AND BIOHYDROGEN PRODUCTION FROM MUTANT STRAINS OF *AZOTOBACTER VINELANDII* DEREGULATED FOR NITROGEN FIXATION

A THESIS SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY

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Abstract

The increase in demand for food and fuel as a result of an increasing population must be sustainable and renewable in the face of global climate change. *Azotobacter vinelandii*, an aerobic nitrogen fixing bacterium, has the potential to supplement or replace a major consumer of global energy, which is the production of ammonium (NH_4^+) for use in fertilizers. A requisite by-product of nitrogen fixation includes the production of hydrogen gas (H_2), which can be used for many applications, including renewable hydrogen fuel cells. *A. vinelandii* produces both of these in a biochemical process which takes place at ambient temperatures and pressures using renewable carbon sources for energy. Within this research, improvement of the conditions needed for higher NH_4^+ and H_2 production from a strain deregulated for the production of nitrogenase was explored and H_2 output was characterized as a result of multiple genetic modifications and changes to culture conditions.

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Chapter 1

Optimization of growth conditions for ammonium production from mutant *Azotobacter vinelandii* strain deregulated for nitrogen fixation

Abstract

The obligate aerobe *Azotobacter vinelandii* is a model organism for the study of biological nitrogen fixation (BNF). This bacterium regulates the process of BNF through the two component NifL and NifA system, where NifA acts as an activator while NifL acts as an anti-activator based on various metabolic signals within the cell. Disruption of the *nifL* component in the *nifLA* operon in a precise manner results in a deregulated phenotype that produces levels of ammonium that far surpass the requirements within the cell, and results in the release of up to 30 mM of ammonium into the growth medium. While many studies over the past eight decades have probed the factors affecting growth of *A. vinelandii*, the features important to maximizing this high-ammonium-accumulating phenotype have not been fully investigated. In this work, we report the effect of temperature, medium composition, and oxygen requirements on sustaining elevated levels of ammonium production from this important strain, providing an analysis of the additional factors required by the cell to sustain this phenotype *in vivo*. We further investigated several pathways, including hydrogen gas recycling and ammonium uptake through the transporter AmtB, which could limit yields through energy loss or futile recycling steps. These findings provide important process optimization parameters, and illustrate further improvements to this phenotype that can be accomplished by eliminating competing cycles. The results further demonstrate that *A. vinelandii* could serve as a platform chemical producer, yielding both hydrogen and ammonium, without a significant loss to ammonium accumulation.

Introduction

Azotobacter vinelandii is a diazotrophic (nitrogen fixing), obligate aerobe studied extensively as a model organism for biological nitrogen fixation (BNF), a complex and energy-intensive process requiring stringent regulation by the cell (1-4). BNF is tightly regulated due to the energetically expensive nature of the reaction which consumes a minimum of 16 mols of ATP and 8 mols of electrons per mol of N_2 fixed (4, 5). Primary expression of the genes encoding nitrogenase in *A. vinelandii* is regulated by the *nifLA* operon clustered in a region of the genome that is located a significant distance from the *nifHDK* genes that encode the catalytic subunits of nitrogenase (NifH and NifDK) (6-8). Previous evidence suggests that NifA acts as an activator of nitrogenase expression, while NifL acts as an anti-activator, sensing available nitrogen, intracellular redox status, and carbon availability within the cell (9). Several laboratories have disrupted the *nifL* gene in *A. vinelandii*, resulting in deregulation of nitrogenase expression and subsequent extracellular ammonium (NH_4^+) accumulation reaching up to 30 mM in the supernatant in the late exponential and stationary phases of growth (6, 10-14). Though much has been elucidated in relation to the nature of deregulation of BNF, there remains many aspects of this NH_4^+-accumulating phenotype which could serve to further our understanding of BNF, and how the cell restructures metabolism during this process.

Our laboratory has reconstructed our own version of this *nifL* deletion resulting in high- NH_4^+ accumulation; *A. vinelandii* strain AZBB163 (11). This deregulated strain is a powerful tool that can be used to probe the overall changes that occur when *A. vinelandii* redirects substantial amounts of energy and resources toward the primary goal of BNF as a result of uninhibited expression of nitrogenase. We recently completed a global transcriptomic study during peak NH_4^+ production in this deregulated strain, which confirmed dramatic increases in the transcription of nitrogenase genes (6), and also provided a glimpse of how the cell regulated multiple other supporting pathways during NH_4^+ accumulation. This study was important, because it provides an opportunity to study a state that is likely transient in typical diazotrophic growth (6, 15).

A. vinelandii has been studied for many decades in relation to the features associated with diazotrophic growth of the wild-type strain (1, 16-18). Under diazotrophic culture conditions, BNF is balanced and tightly regulated, assuring that the cell produces only as

much nitrogen as is required to sustain its own growth. Factors that affect the rate of BNF in wild-type cells are generally indirectly measured through growth rate or using non-native substrates such as acetylene, that result in a terminal product that can be easily quantified (19, 20). Deregulated strains of nitrogen fixing bacteria such as AZBB163 have been reprogrammed to express nitrogenase regardless of internal nitrogen requirements, and could be a valuable tool in determining the specific factors that might limit BNF in this phenotype. Based on the potential applications of these deregulated strains as an alternative for producing high levels of fixed nitrogen, and the opportunity to measure production directly by monitoring levels of NH_4^+ accumulation in spent culture medium, we believed it was important to characterize the different factors that could be limiting production of NH_4^+ in AZBB163. These studies include environmental parameters related to the culture conditions alone and in tandem with additional genomic modifications. We further investigated potential competing pathways that we hypothesized would become exacerbated under the conditions associated with the high NH_4^+ accumulating phenotype, and might result in energetically costly and futile cycles that inadvertently limit BNF, such as the NH_4^+ importer, AmtB (21, 22). A description of these parameters and pathways that were targeted, and the findings associated with them, are presented below, and demonstrate a potential to further improve this process and the features of the strain, and additionally yield multiple products that could further enhance the economic potential of pursuing routes to biofertilizer production through BNF (23-25).

Materials and Methods

Genetic Constructs. *Azotobacter vinelandii* DJ (ATCC BAA-1303) was obtained from Dennis Dean (Virginia Tech) and cultured on Burk's medium (26). *Escherichia coli* JM109 was acquired from New England Biolabs (Ipswich, MA) and utilized to construct the plasmids described. Plasmids used for constructing final vectors for gene deletions in *A. vinelandii* are described in Table 1.1. Primers used for cloning and confirmation are described in Table 1.2. Detailed methods for the transformation of *A. vinelandii* have been described previously (26, 27). Methods describing the construction of strain AZBB163 have been described previously (11).

Gene Deletion and Confirmation. Transformation of *A. vinelandii* DJ in order to modify target genes utilized double homologous recombination and the *pyrF* counter-selection technique as described previously (11, 27). Strains generated and used in this study were constructed as described in Table 1.3. Strain AZBB163 was constructed as described previously (11).

Growth of Cells and B/MoFeS Medium development. *A. vinelandii* strains AZBB163 and AZBB281 were grown in acid-base washed 125 mL Erlenmeyer flasks containing 60 mL of medium unless otherwise stated. Experiments measuring the effect of increased aeration were performed by inoculating a larger volume of sterile medium, and then adding aliquots of different volumes (15 ml, 30 ml, or 60 ml) of inoculated medium into sterile flasks of the same size (125 mL). All experiments were performed with at least 3 replicates. Burk's/ Na_2MoO_4, $FeSO_4$, Na_2SO_4 (B/MoFeS) medium was developed using standard Burk's (B) medium recipes (26) supplemented with 2x iron (18 µM increased to 36 µM as $FeSO_4$), 5x molybdenum (1 µM increased to 5 µM as Na_2MoO_4), and 3x sulfur (0.8 mM increased to 2.4 mM as Na_2SO_4) as determined through a series of experiments testing ranges of these elements. In each experiment, cultures were inoculated with approximately equal initial concentrations of cells within each particular experiment from cells scraped off B plates containing appropriate antibiotics grown for 2 days at

30°C. Cultures were grown at 26°C with agitation at 180 rpm unless otherwise noted. The temperature of 26°C was chosen as a standard due to the balance of ammonium (NH_4^+) produced per culture density, which was maximal at this temperature. Supernatant was collected by centrifugation (~20,000 x g for 30 seconds) of samples at the indicted time points.

Ammonium Quantification. Assays for NH_4^+ quantification used the colorimetric o-phthalaldehyde method as described previously utilizing a Cary 50 Bio Spectrophotometer measuring absorbance at 412 nm (6).

Sucrose Quantification. For experiments measuring the levels of sucrose in the medium, the Sigma-Aldrich kit (Part No: MAK013-1KT) was used as directed by the manufacturer.

Protein Quantification. To quantify the total amount of starting protein, isolated cell pellets were suspended in one mL of water and sonicated for 60 seconds (Misonix LX-2000, Qsonica, Newtown, CT) inside of a 2.0 mL Eppendorf tube, and then centrifuged at 13,000g for 5 minutes to remove cell debris. Cell lysate was then added to 1.0 mL of Coomassie Plus (Bradford) assay reagent (Pierce, Rockford, IL), mixed by pipetting and incubated at room temperature for 20 minutes. Absorbance was read at a wavelength of 595 nm (Varian Inc., Palo Alto, CA). Samples were compared to a standard curve prepared using Bovine Serum Albumin as a standard (Pierce, Rockford, IL).

Table 1.1. Plasmids and plasmid derivatives used in this study

Plasmid[a]	Relevant gene(s) Cloned or Plasmids Manipulated[b]	Parent Vector	Source or Reference
pBB053	Removed NdeI site from pUC19 by silent mutation	pUC19	(28)
pPCRAMTBK2*	Removal of *amtB* from parent vector via PCR for use in deleting *amtB* from *A. vinelandii*	pPCRAMTBK1	(27)
pPCRAMTBK4*	Insertion of Kan^R and *pyrF* cassette between the flanking regions of *amtB* in *A. vinelandii* to be used for marker-less deletion of *amtB*	pPCRAMTBK2	(27)
pPCRHYDK7	Combined two regions flanking $hoxN_1IN_2YHW$ from pPCRHYDK3 and pPCRHYDK4 into new vector	pPCRHYDK3	(6)
pPCRHYDK8	Combined two regions flanking *hoxKGZMLOQRTV* from pPCRHYDK5 and pPCRHYDK6 into new vector	pPCRHYDK5	(6)
pPCRHYDK9*	Insertion of kan^R and *pyrF* cassette between the flanking regions of $hoxN_1IN_2YHW$ for deletion via *pyrF* counter selection.	pPCRHYDK7	This study
pPCRHYDK10*	Insertion of kan^R and *pyrF* cassette between the flanking regions of *hoxKGZMLOQRTV* for deletion via *pyrF* counter selection.	pPCRHYDK8	This study
pPCRKAN15	kan^R and *pyrF* cassette used for insertion for *pyrF* and counter selection.	pPCRKAN11	(27)
pPCRNH3-43*	Removed large segment of *nifL* gene and inserted Kan cassette upstream of *nifA*	pBB053	(11)
pPCRNH3-44*	Incorporated C to T mutation into kanamycin cassette region to create Nif^+ phenotypes with high ammonium production	pPCRNH3-43	(6)
pPCRPYRF1*	Cloned *pyrF* from *A. vinelandii*	pBB053	(27)
pPCRPYRF3	Removal of *pyrF* from parent vector via PCR for use in deleting *pyrF* in *A. vinelandii*	pPCRPYRF1	(27)

[a] The sequences of all plasmids in this study are available upon request. Plasmids indicated by an asterisk (*) are completed vectors that were used to transform *A. vinelandii*.

b Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance.

N_1 = Avin_04360 and N_2 = Avin_04380

Table 1.2. Primers used in this study

Primer	Sequence 5' – 3'	Purpose
BBP950	GAGCACACCCATCACGGTCAGAG	*nifLA* modification confirmation
BBP1322	GATCTCCATCGACTCGATCTTGTCCAGGGTGAAC	*nifLA* modification confirmation
BBP1862	NNNTCTAGA GTGGTCTTCGTCCTGGCCGACGAG	*pyrF* gene cloning
BBP1863	NNNAAGCTT CGAAGCGCAGCGCGACGACCTCCAG	*pyrF* gene cloning
BBP1866	NNNAGATCT CGAACTGGACTGAGTGCGTTC	*pyrF* gene deletion
BBP1867	NNNAGATCT CTAGGACAAATGTTCCAGGGCGAAG	*pyrF* gene deletion
BBP1883	CGATCACCGAGCCAAACACCACTATCAG	*pyrF* gene deletion confirmation
BBP1884	CGCTGGTCGTTGCACCAACTCGGATGAG	*pyrF* gene deletion confirmation
BBP1969	NNNAGATCT GGTTACAACCTCTGAGTGTCGGGAG	*amtB* gene deletion
BBP1970	NNNAGATCT CAGCGTCATTGATTATTCTCCTGGGGCG	*amtB* gene deletion
BBP2006	CACGTGCCAGGAATTCCTCCATG	*amtB* gene deletion confirmation
BBP2007	CTGTGGACGATGGCCAGGGACATGGATC	*amtB* gene deletion confirmation
BBP2247	GATAGCCCAGTAGCTGATATTCATCCGGATCATCC	C to T mutation resulting in Nif+ phenotype found in strain AZBB163
BBP2248	GGATGATCCGGATGAATATCAGCTACTGGGCTATC	C to T mutation resulting in Nif+ phenotype found in strain AZBB163
BBP2395	GTCTGCCAGG ACTTCGCCCA CCTGATGCTC	*hox*N_1*IN*$_2$*YHW* deletion confirmation
BBP2396	CAGCGACGGC GTGGTCAGGT ACAGGTC	*hox*N_1*IN*$_2$*YHW* deletion confirmation
BBP2397	GTCGTCCAGC ACGGTCAGTG GATGACAG	*hoxKGZMLOQRTV* deletion confirmation
BBP2398	GCTGACGCTC GATCCAGGCC AGCCACTC	*hoxKGZMLOQRTV* deletion confirmation

N_1 = Avin_04360 and N_2 = Avin_04380

Table 1.3. Key strains constructed and utilized in this study

A. vinelandii Strain	Genetic Features	Plasmid utilized	Parent strain
AV Trans	Wild-type A. vinelandii with diminished alginate production resulting in ease of transformation	none	None
AZBB072[c]	Δ*pyrF*	pPCRPYRF3	AV Trans
AZBB109	Δ*amtB*	pPCRPYRF1	AZBB106[c]
AZBB163[b]*	*nifL::kan*^{R}-(pPCRNH3-43). Was grown in medium without urea, and mutated to a nif+ phenotype that also produces high levels of extracellular ammonia.	pPCRNH3-43, spontaneous mutation	AZBB150[b]
AZBB198	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*::*pyrF*-kan^R	pPCRHYDK9	AZBB105[c]
AZBB212	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*	pPCRHYDK7	AZBB198
AZBB219	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*, *hoxKGZMLOQRTV*::*pyrF*-kan^R.	pPCRHYDK10	AZBB212
AZBB233	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*	pPCRHYDK8	AZBB219
AZBB261	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*,	pPCRPYRF1	AZBB233
AZBB269*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW (Hydrogenase Region 1),* Δ*hoxKGZMLOQRTV (Hydrogenase Region 2), nifL::kan-pPCRNH3-44 col 2.*	pPCRNH3-44	AZBB261
AZBB281*	Δ*amtB, nifL*::kan-pPCRNH3-44	pPCRNH3-44	AZBB109

[a] Strep^R: Streptinomycin, Kan^R: Kanamycin resistance.
[b] As described previously (11)
[c] As described previously (27)
N$_1$ = Avin_04360 and N$_2$ = Avin_04380
Strains indicated by an asterisk (*) are completed and were used in ammonium production experiments.

Results

The effect of varied temperature on ammonium production. Ammonium (NH_4^+) levels achieved by cultures grown under different temperatures varied significantly, with 28°C resulting in the highest NH_4^+ concentration per culture, and 26°C resulting in the highest NH_4^+ concentration per culture density (Figure 1.1). OD_{600} values were not significantly different between the samples grown at 24 and 26°C, but 28 and 30°C resulted in cultures that maintained a significantly higher OD_{600}. Interestingly, while total NH_4^+ levels increased steadily from 24 to 28°C, 30°C resulted in a steep decrease in the achieved NH_4^+ concentrations.

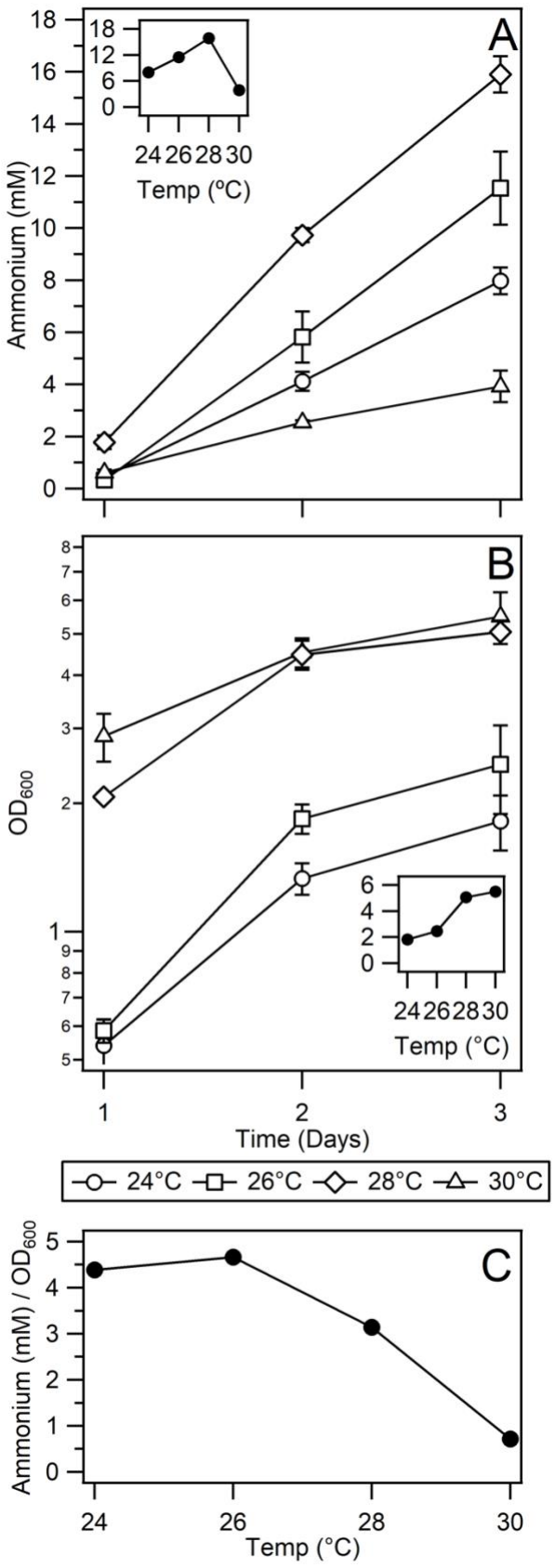

Figure 1.1: Ammonium production and OD_{600} over time in AZBB163 grown at varied temperatures. Shown are the ammonium (NH_4^+) production (Graph A), OD_{600} (Graph B), and NH_4^+ per OD_{600} on day 3 of growth (Graph C) results from AZBB163 grown in Burk's (B) medium at 24, 26, 28, and 30°C. Inset in Graph A represents NH_4^+ values for each temperature on day 3 and inset in Graph B represents OD_{600} on day 3 of the experiment. Cultures were shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=4).

The effect of increased metals and sulfur concentrations on NH_4^+ production. In an effort to ameliorate the potential limitations of essential elements required for cofactor assembly related to nitrogenase, a supplemented medium was developed via a series of experiments testing increases in molybdenum, iron and sulfur (B/MoFeS); elements essential to metal cluster assembly for nitrogenase component proteins (4). These preliminary experiments established levels that increased availability without inducing a detrimental response due to potential toxicity. The adapted medium (B/MoFeS) balanced an increase in NH_4^+ production with a minimal increase in the concentration of each of these three elements, resulting in the final modified medium containing sulfur increased 3-fold, molybdenum 5-fold, and iron 2-fold as compared to standard B medium. AZBB163 was grown in this medium modified for high levels of nitrogenase expression (Figure 1.2). The results demonstrate a statistically significant ~25% increase in the amount of NH_4^+ produced on days 2 and 3 of growth, while there was a significant decrease in the OD_{600} on days 1 and 2 in the presence of supplemented cofactor-related elements ($p \leq 0.05$).

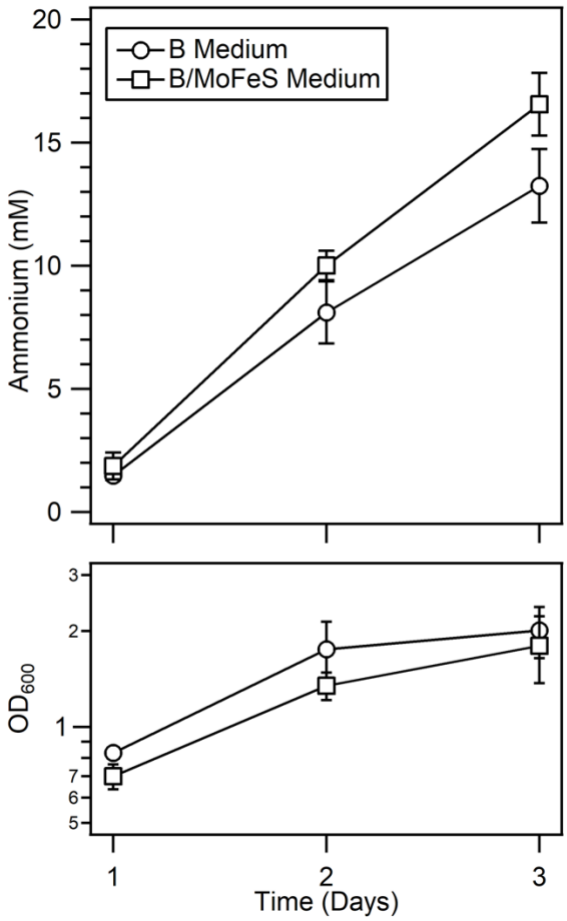

Figure 1.2: Ammonium production and OD_{600} over time in AZBB163 grown standard B medium and B/MoFeS medium. Shown are the ammonium production (top) and OD_{600} (bottom) results from growing AZBB163 in both standard Burk's (B) medium and B medium supplemented with nitrogenase cofactor-related elements (2-fold increase in iron (Fe), 5-fold increase in molybdenum (Mo), and 3-fold increase in sulfur (S)). Cultures were grown at 26°C and shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=5).

Sucrose consumption and limitation in AZBB163. An additional goal within this work was to profile the rate of sucrose consumption in AZBB163 during production of extracellular NH_4^+ as a means of correlating NH_4^+ produced to required energy. Varied concentrations of sucrose in B/MoFeS medium were tested in growths of AZBB163, and NH_4^+ levels were analyzed to determine when specific cultures would plateau in NH_4^+

production (Figure 1.3). B/MoFeS medium was utilized to ensure that sucrose, and not a shortage in the elements related to co-factor assembly, was the limiting factor for growth and NH_4^+ production. Cultures containing 2 g/L became limited for sucrose after 1 day of growth, whereas cultures grown in 5 g/L of sucrose were found to plateau in NH_4^+ production after 2 days of growth. The cultures containing 10 g/L of sucrose and 20 g/L sucrose (the typical concentration in B medium) were not significantly different in either NH_4^+ concentrations achieved or culture density on all 3 days of the experiment. In order to more accurately profile the rate of sucrose consumption within this strain that is deregulated for nitrogenase expression, initial sucrose concentrations in B/MoFeS medium were lowered to 10 g/L and cultures were grown beyond the point of sucrose depletion (Figure 1.4). Similar to the previous experiment profiling sucrose consumption at varied concentrations, B/MoFeS medium was utilized. After 4 days, nearly all sucrose was consumed from the medium, and after 3 days, increases in NH_4^+ concentration had plateaued.

The highest rates of NH_4^+ production and sucrose consumption occurred between 12 and 72 hours. The relationship between mmols of NH_4^+ produced per mmol of sucrose consumed was nearly 1:1 in this time frame. Under these conditions, the cultures were inoculated into medium containing approximately 30 mM of sucrose and ceased accumulation of NH_4^+ at concentrations of 20 mM, making the net yield approximately 1.5 mol sucrose consumed per mol NH_4^+ produced. It is worthwhile to note that once the culture depleted the sucrose from the medium, NH_4^+ levels remained steady for several days of further culturing under sucrose depletion.

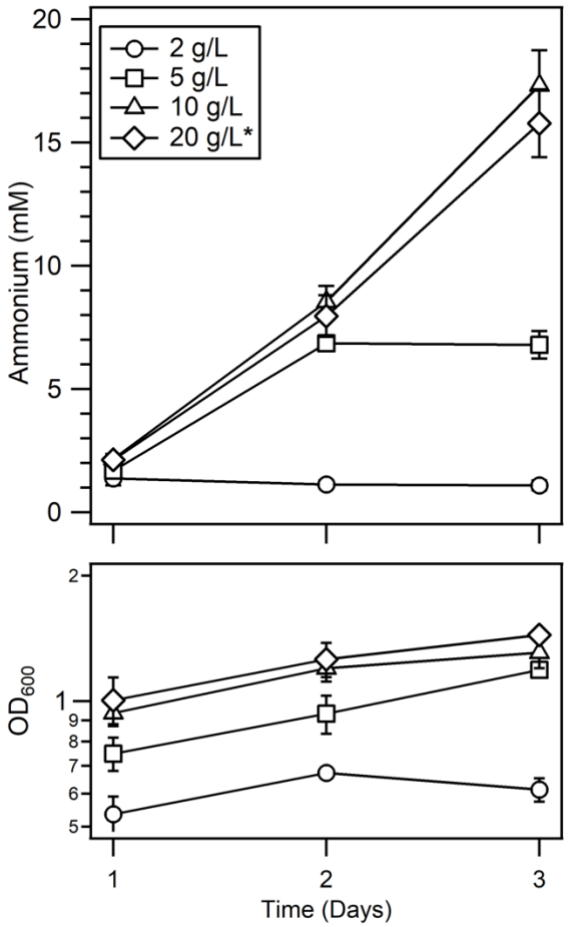

Figure 1.3: Ammonium production and OD_{600} over time in AZBB163 grown in B/MoFeS medium with varied concentrations of sucrose. Shown are the ammonium production (top) and OD_{600} (bottom) results from growing AZBB163 in Burk's (B) medium supplemented with cofactor-related elements (2-fold increase in iron (Fe), 5-fold increase in molybdenum (Mo), and 3-fold increase in sulfur (S)) with varied sucrose concentrations, with the standard concentration of sucrose (20 g/L) indicated with an (*). Cultures were grown at 26°C and shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=4).

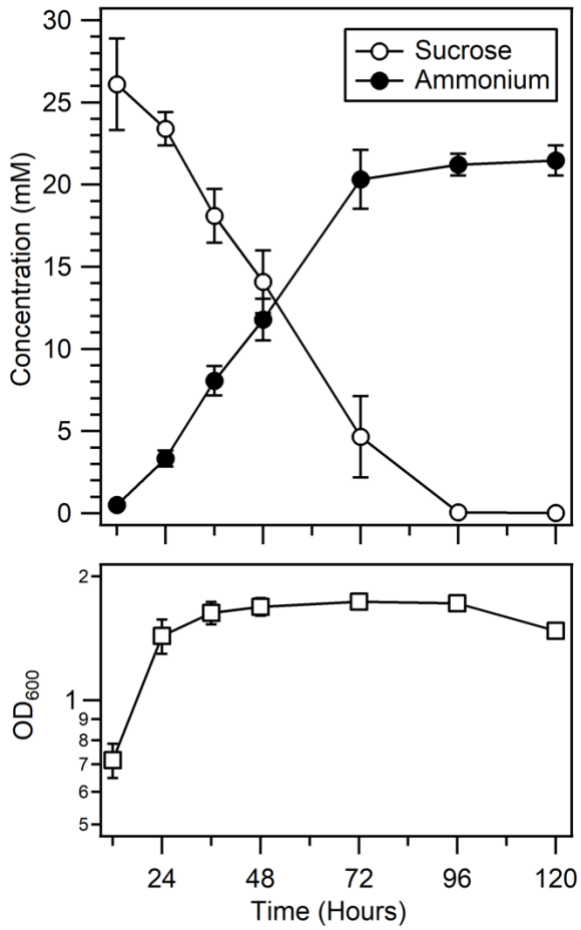

Figure 1.4: Ammonium production, OD_{600}, and sucrose consumption over time in AZBB163 grown in B/MoFeS medium. Shown are the ammonium production and sucrose consumption (top) and OD_{600} (bottom) results from growing AZBB163 in Burk's medium supplemented with cofactor-related elements (B/MoFeS medium) with an initial sucrose concentration of 10g/L. Cultures were grown at 26°C and shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=7).

The effect of culture volume on NH_4^+ production. *A. vinelandii* is an obligate aerobe, and given the high ATP demand from AZBB163 as a result of excess BNF, oxygen availability may be limiting growth. A simple approach to increase oxygen availability in batch culture is to decrease the total culture volume within the flask while keeping the flask size the same, providing a greater proportion of exposed surface area to atmosphere

versus the volume of culture and more vigorous mixing. NH_4^+ production was tested in cultures with volumes of 15 mL, 30 mL, and 60 mL each in 125 mL Erlenmeyer flasks (Figure 1.5). All three volumes yielded similar concentrations of NH_4^+ up to 16 hours. However, between 16 and 32 hours, the cultures significantly deviated with respect to NH_4^+ concentration, with the cultures containing 15 mL of medium producing the highest concentrations of NH_4^+, followed by the 30 mL cultures, and finally the 60 mL cultures. All conditions resulted in similar culture densities up to 8 hours of growth, after which the 15 mL cultures grew more densely, while the 30 mL and 60 mL cultures maintained a lower OD_{600}. While evaporation rates within cultures of smaller volumes was a concern, control experiments revealed that there was a negligible decrease in culture volume over time due to evaporation, indicating that the differences in NH_4^+ levels were independent of changes in culture volume based on the experimental design.

Alternative experiments that employed baffled flasks to further increase the aeration resulted in a sharp drop in levels of NH_4^+ achieved, and increased foaming. These results indicate that alternative approaches to increase oxygen availability should be approached with caution.

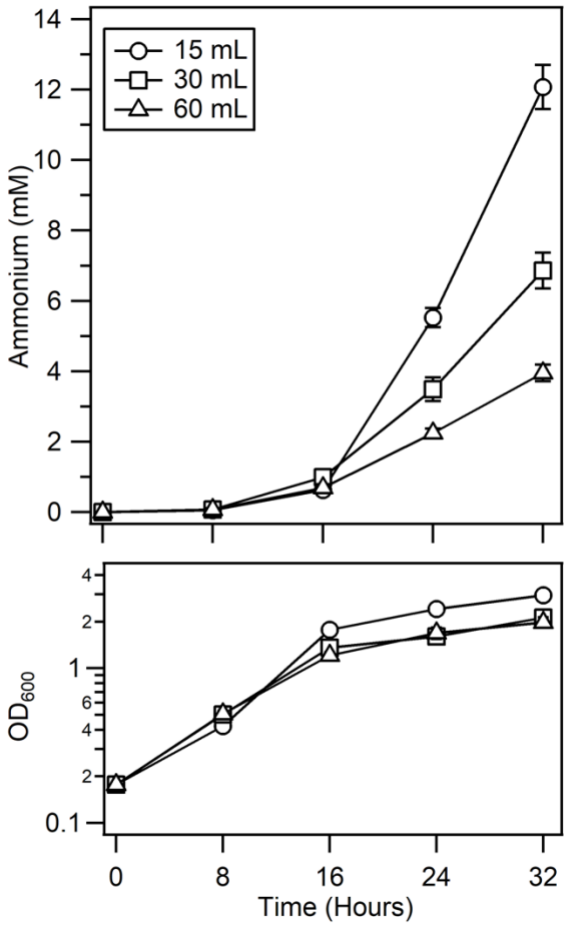

Figure 1.5: Ammonium production and OD_{600} over time in AZBB163 in cultures with varied volumes. Shown are the ammonium production (top) and OD_{600} (bottom) results from growing AZBB163 in Burk's (B) medium in flasks of the same size but with varied volumes of culture added. Cultures were grown at 26°C and shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=5).

NH_4^+ production in AZBB163 lacking membrane bound and soluble hydrogenases. A natural extension of this research involving NH_4^+ production, is to explore the other key product of BNF, which is H_2 gas. In a strain deregulated for BNF and producing copious amounts of NH_4^+, excess hydrogen gas would also be produced. However, the uptake hydrogenase would need to be deleted in such a strain in order for this produced hydrogen gas to be free to escape and not be recaptured for use by the cell (6, 29, 30). Within this research, *pyrF* counter selection was used to delete both the membrane bound

and soluble hydrogenases, and the ability of the cell to still fix nitrogen at high levels was assessed. After three days of growth in standard B medium, there was no significant difference in either NH_4^+ production or culture density between the cultures that either contained or lacked the uptake hydrogenase that has been cited as playing an important role in hydrogen recycle (6, 29, 30). From this result, it is shown that within this phenotype, and under these growth conditions, the hydrogenases did not serve an important role in maintaining the NH_4^+ levels in these strains.

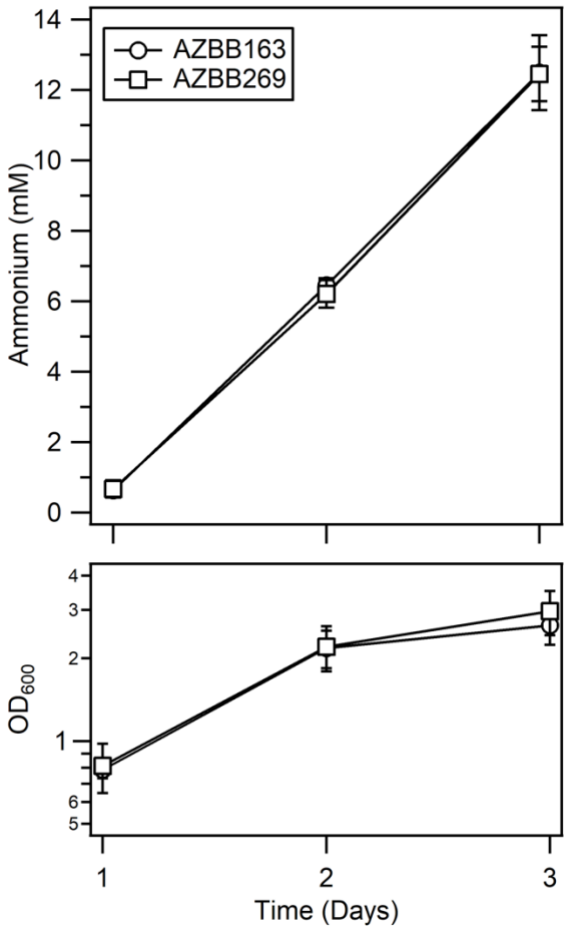

Figure 1.6: Ammonium production and OD_{600} over time in high ammonium producing strains lacking both the membrane-bound and soluble hydrogenase. Shown are the ammonium production (top) and OD_{600} (bottom) of AZBB163 and AZBB163 lacking both the soluble hydrogenase and the membrane bound hydrogenase (AZBB269). Cultures were grown in Burk's (B) medium at 26°C and shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=4).

Disruption of futile cycles in NH_4^+ import. The NH_4^+ transporter AmtB is constitutively expressed in *A. vinelandii*, regardless of extracellular NH_4^+ levels (6). In a strain such as AZBB163 that has been deregulated for BNF, this represents a potentially futile and energy wasting cycle (Figure 1.7). We hypothesized that if the *amtB* gene were deleted, it might result in a higher level of NH_4^+ accumulation within the supernatant. Mimicking previous studies (11, 27), AmtB was deleted in AZBB163, creating *A. vinelandii* strain AZBB281. When evaluated for NH_4^+ production, our results demonstrated that AZBB281 accumulated elevated levels of NH_4^+ in the supernatant as compared to AZBB163 at both day 2 and day 3 (Figure 1.7), illustrating that this process may represent an energetically wasteful process.

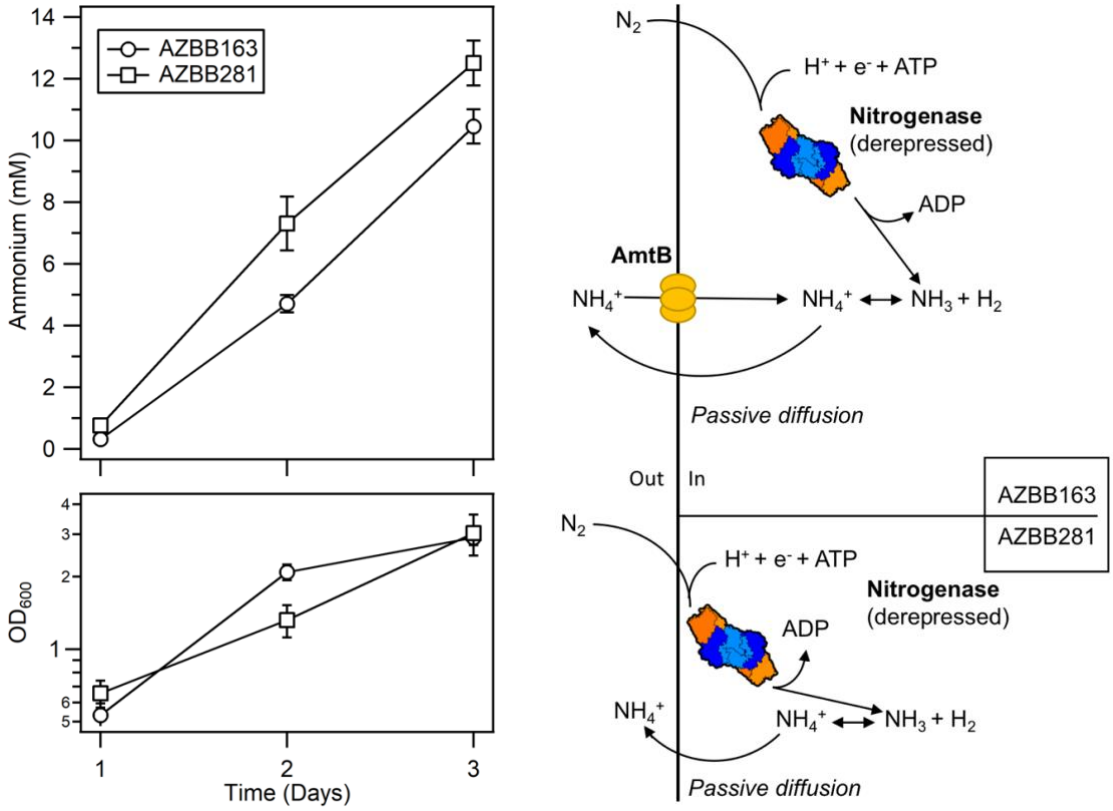

Figure 1.7: Ammonium production and OD600 over time in AZBB163 and AZBB281. Shown are the ammonium (top left) and OD_{600} (bottom left) results of growing AZBB163 (high ammonium producing strain) and AZBB281 (*amtB* deletion in strain containing

AZBB163 mutation) in standard B medium. Shown on the right is a cartoon representation of the effects of deleting *amtB* (the unidirectional ammonium importer) within a strain of Azotobacter deregulated for nitrogen fixation. ”In” and “out” refer to the intracellular and extracellular sides of the cellular membrane, respectively. Cultures were grown at 26°C and shaken at 180 rpm. Data points indicate averages, while the error bars represent standard deviation. N=4.

Incorporation of constructs and conditions found beneficial to NH_4^+ production per culture. As a final experiment, we sought to incorporate all of the augmented conditions that resulted in improved NH_4^+ per culture with the strain lacking the futile cycle for NH_4^+ uptake to compare the increases that could be achieved (Figure 1.8). Strain AZBB281 was grown at 28°C, in 15 mL of B/MoFeS medium. These combined conditions yielded both high OD_{600} values and the highest rate of NH_4^+ production from any of these experiments. During the eight-hour period between 24 and 32 hours, rates of approximately 0.95 mM NH_4^+ per hour were achieved. This value is equivalent to nearly 16 μM per minute (16 nmol/min per mL). Protein levels between 24 and 32 hours averaged about 412 μg/mL (323 and 502 μg/mL at 24 and 32 hours, respectively). To yield 16 nmol/min of NH_4^+ with purified nitrogenase enzyme requires approximately 27 μg of MoFe protein (NifDK) with an excess of Fe protein (NifH) (31, 32). Based on these calculations, 5-8% of the cellular protein is required as MoFe protein (NifDK) functioning at optimal rates (10 molar equivalents of Fe protein to MoFe protein (32)).

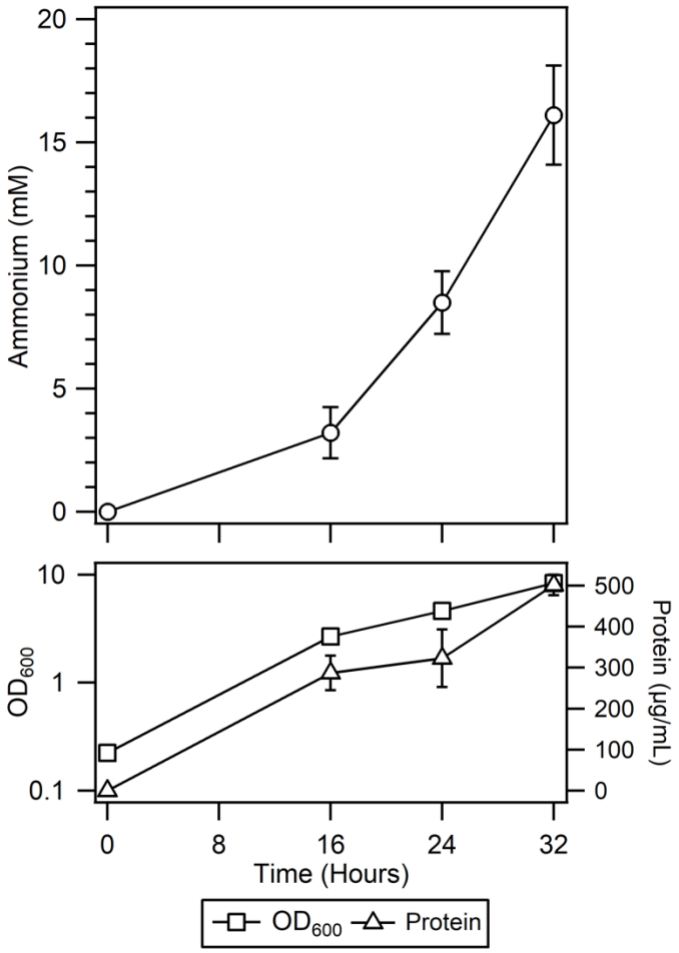

Figure 1.8: Ammonium production and protein yield in AZBB281 grown under all conditions found to improve ammonium production per culture. Shown are the ammonium (NH_4^+) production (top), protein and OD_{600} (bottom) of AZBB281 grown under the all conditions found to produce the most NH_4^+ per culture. Cultures were grown at 28°C, with 15 mL culture volumes, in Burk's medium supplemented with additional nitrogenase cofactor-related elements (B/MoFeS medium), and shaken at 180 rpm. Data points indicate averages, while error bars represent standard deviation (N=5).

Discussion

Optimization of growth conditions to maximize the production of desired products is a simple, yet robust way to accomplish what more difficult genetic manipulations may only potentially achieve. Both culture conditions and additional genetic manipulations were used independently and in tandem to yield further increases in the accumulation of NH_4^+ in the spent medium of *A. vinelandii* strains that had been deregulated for BNF. These findings provide insight into the limiting factors that *A. vinelandii* may face when deregulated for BNF and producing copious quantities of NH_4^+. The growth parameters investigated in this study include oxygen limitations, temperature, and availability of the Mo, Fe and S used for the biosynthesis of cofactors essential for nitrogenase. One aspect that became evident as a result of these findings is that there is a balancing act that must be taken into consideration when seeking to optimize conditions for deregulated BNF.

Modifying the temperature at which the cultures were grown had a profound and interesting effect on the amount of NH_4^+ produced. The highest temperature tested (30°C) was found to be detrimental to NH_4^+ accumulation versus slight decreases in culture temperature, and resulted in a trade-off in limiting NH_4^+ while yielding an increase in culture density (Figure 1.1). Growing cultures at a slightly lower temperature of 28°C resulted in the highest concentrations of NH_4^+ accumulation in the medium, which was also accompanied by the elevation of culture density. Lowering the temperature even further to 26°C resulted in similarly high NH_4^+ levels, but dramatically lowered culture density, and growth at 24°C gave similarly low culture density, but lower levels of NH_4^+, while still achieving higher levels of NH_4^+ than cultures grown at 30°C. Ideal and typical conditions for growing *A. vinelandii* are often cited as 30°C in the primary literature (33-36), and 30°C is the suggested growth parameters when the wild-type strain is obtained through the American Type Cultures Collection (ATCC).

This suggested temperature of 30°C for culturing *A. vinelandii* differs from optimal conditions for respiration described by Lineweaver, Burk and Horner more than 80 years ago, who found that *A. vinelandii* respires at increasingly higher rates up to 35°C, after which point respiration rates decrease (1). The effect of temperature on BNF in various

bacteria has been studied previously by probing a wide range of temperatures using the alternative substrate acetylene, which is often selected for measuring intracellular nitrogenase activity due to the ease of performing these assays, and found optimal rates of activity at 35°C for *Azotobacter choococcum* (37). Additional studies have looked at the regulation of nitrogenase related gene expression at different temperatures in *A. vinelandii* (38, 39). In general, the activity of isolated nitrogenase enzymes tends to increase with steadily higher temperatures through 45°C (40, 41). Since most species of *Azotobacter* produce only as much NH_4^+ as is necessary to sustain their own growth, and since many assays of nitrogenase activity done *in vivo* actually use the substrate acetylene, which has characteristics that differentiate it from N_2, the strain in our study provides a unique means of testing the effect of temperature on BNF in a strain deregulated for this process, and yielded an optimal temperature for NH_4^+ production that contrasts with what has been reported in these previous studies. This is not completely unexpected, as the metabolism within this strain has been altered dramatically to achieve this NH_4^+ production phenotype.

The shift in cell density that occurred between 26 and 28°C was also an interesting curiosity (Figure 1.1). We previously reported differences in final cell densities that were obtained between the AZBB163 and the wild-type strain (6). The stark change that occurred in AZBB163 during this rather narrow temperature range could result from several different sources, including a shift of metabolism that favors polyhydroxybutyrate production or a limitation of other key intracellular metabolites. However, it clearly indicates a transition in the metabolism that is occurring through this narrow temperature transition, and the result of that transition is a dramatic decrease in NH_4^+ production between 28 and 30°C. This specific feature of the strain could be investigated further in the future using different techniques, but at this point in time, further investigation was outside the scope of this study.

Increased cell density should translate to increased competition for available oxygen. It is well established that *A. vinelandii* expends a great deal of energy attempting to carefully modulate internal oxygen levels (1, 42). Since oxygen solubility in water is actually quite low versus many other gases, and *A. vinelandii* is known to have one of the highest respiratory rates reported for bacteria (1, 18), it was quite possible that oxygen

availability could be limiting BNF and the excess NH_4^+ production that results in the NH_4^+-releasing phenotype that is associated with AZBB163. In contrast, nitrogenase is an oxygen-sensitive enzyme, and elevated levels of oxygen near the active site of nitrogenase could also irreversibly damage this enzyme. This very interesting phenomenon of these two highly dependent processes seems to be at odds with one another, and the AZBB163 strain also offered a unique opportunity to determine how well the cell protects these elevated levels of nitrogenase under these conditions and whether the strain was in fact oxygen limited as a result.

Outside of using a fermenter or other aeration device, there are several manners by which oxygen delivery could be improved to batch-grown flask cultures. One would be to increase the fraction of oxygen within the atmosphere. However, since *A. vinelandii* also needs the nitrogen from the atmosphere in these experiments to measure BNF, this was thought to be a self-defeating approach. The addition of baffles to the Erlenmeyer flask was also viewed as a favorable method of introducing more oxygen, but resulted in significant foaming and aggregation of the cells, accompanied by a dramatic drop in NH_4^+ production. Reduction of culture volume, while keeping the flask size constant, is a classical way to provide more oxygen to batch grown cultures by increasing the surface area exposed to air, resulting in increased aeration (43). The approach of lowering the volume of culture while maintaining the same size of Erlenmeyer flask proved to be the most beneficial to test this phenomenon, and the results clearly indicate that the NH_4^+ production could be steadily and significantly increased by lowering the ratio of volume versus the available surface area exposed to the standard oxygen atmosphere (Figure 1.5). This illustrates that the culture is still oxygen limited under our standard, starting conditions, and that the strain is well adapted to protect the nitrogenase from any toxic effects associated with the oxygen, even with the elevated expression of nitrogenase that accompanies this phenotype (6, 12).

In a previous study investigating the changes in global transcription that occur within AZBB163 (6), we determined that molybdenum availability was limiting NH_4^+ accumulation in AZBB163. The production of the full complement of metal clusters in each full nitrogenase complex (NifDK + 2 NifH) requires 38 Fe and 2 Mo, but also requires 40 S atoms. Each of these clusters is bound to the enzyme through one or more

cysteine ligands, which would also increase the demand for S. Based on the elevated levels of expression of both NifDK and NifH that were found from our prior analysis (6), and the relatively low concentration of sulfate found in the general recipe for Burk's medium (26), we hypothesized that sulfur might also be limiting NH_4^+ production in AZBB163. Experiments revealed that elevating the levels of both Mo and S, independently and in combination, resulted in a noticeable increase in NH_4^+ accumulation. Based on these observations, a new medium was formulated that increased levels of Mo by 5-fold, Fe by 2-fold and S by 3-fold. Further increases to the concentrations of each element individually resulted in no further improvement, or a noticeable drop in NH_4^+ accumulation, including for elevated levels of S. The optimized medium did result in an increase in NH_4^+ accumulation as compared to standard Burk's medium (Figure 1.2). Though the original Burk's medium recipe does provide sufficient levels of these microelements to maintain the diazotrophic phenotype for the wild-type strain, the elevated requirements of AZBB163 to sustain elevated NH_4^+ production necessitates an increase in sulfur as well as the previous levels of Mo that were required, regardless of the ability of the cell to recycle such nutrients. This rationale is supported by our previous findings for AZBB163, indicating a limitation due to metal depletion by the cell, which manifested as an increase in transcription for siderophore peptides (6, 44), but diminished upon addition of excess metals.

Hydrogenases are generally associated with BNF, as the process of BNF yields stoichiometric quantities of H_2 as a byproduct of the nitrogenase reaction (30, 45, 46). Hydrogenase is able to recycle the electrons from this process to conserve energy. For this reason, we selected to evaluate the effect on NH_4^+ accumulation if the hydrogenase was not present in the AZBB163 parent strain. Deletion of the two hydrogenase clusters from *A. vinelandii* effectively eliminates the potential of the bacterium to recycle the energy that is released in the form of H_2 gas, and was proposed to result in a less efficient strain when combined with the deregulated nitrogenase strain, as this should create a metabolic stress on the organism. However, while this should result in a wasteful process for the bacterium, this did not decrease the amount of NH_4^+ accumulated to any significant extent when grown under these conditions (Figure 1.6). In previous studies, it has been shown that deletion of hydrogenases within wild-type *A. vinelandii* does not

dramatically decrease rates of growth through disrupted metabolic activity (47), and that the presence of hydrogenase in cells capable of BNF only confers a benefit under conditions of carbon limitation. The question of whether this would affect NH_4^+ production efficiency in a deregulated strain had not been investigated, and our results further indicate that this loss of H_2 is not detrimental to the cell in this strain deregulated for BNF. As a consequence of this finding, this indicates that this newly constructed strain (AZBB269) could be dual-purposed, producing significant amounts of NH_4^+, and also producing H_2 as a secondary product. Through this process, this would transform *A. vinelandii* into a platform species for the production of multiple important chemicals. While the process of H_2 production described above should result in a small quantity of energy released for every molecule of N_2 that is fixed into NH_4^+ ions, the quantity of H_2 produced should be stoichiometrically matched to the quantity of N_2 fixed.

Our previous efforts to yield strains with the potential to serve as a biofertilizer also revealed that elimination of the gene *amtB*, which codes for an NH_4^+ transporter, results in the release of NH_4^+ to the extracellular culture medium that is sufficient to support the co-culture of algae (11). AmtB is thought to serve a role in *A. vinelandii* to shuttle ammonium into the cell by linking this transport to the activity of glutamine synthetase, resulting in the hydrolysis of ATP, which also serves a vital role in BNF (4). It has also been shown that NH_4^+ and methylamine transport in *A. vinelandii* is dependent on membrane potential (48, 49). For this reason, any strains manipulated to produce elevated levels of extracellular NH_4^+ are potentially wasting ATP or energy associated with maintaining the membrane potential in an effort to recycle that NH_4^+ back into the cell in a process that should be futile and wasteful. Additionally, since this process is not directly coupled to BNF, it could result in a much higher waste of energy than what would be expected for deletion of the hydrogenase, especially as levels of NH_4^+ accumulate outside of the cell. To test whether this transporter system was wasting energy and, as a result, hindering the amounts of NH_4^+ that could be accumulated, we also constructed a strain containing the *amtB* deletion (27) along with the modification resulting in the deregulation of BNF. Contrary to what was found for deleting the hydrogenase, the deletion of *amtB* did result in a noticeable improvement in NH_4^+

accumulation (Figure 1.7), indicating that this process is likely wasting unnecessary energy and limiting NH_4^+ accumulation.

The true nature of the transport of NH_4^+ or NH_3 by AmtB remains contested (49-51), and is often tested by using ^{14}C tracers of methylamine to track movement through this transporter (21, 48). Because our deregulated strains are producing elevated levels of both intracellular and extracellular NH_4^+, these strains could serve as a valuable test system to better understand the role of AmtB in the membrane without having to yield artificial systems or incorporate this gene into non-native cells. Though such studies are outside the scope of the work done here, these strains could serve as a valuable tool in the future to better understand the role that AmtB plays in NH_4^+/ NH_3 transport, and to better understand how deletion of *amtB* results in a phenotype that supports the co-culture of dense algae biomass (11).

As a final experiment, we attempted to incorporate all of the improvements that were obtained through both the medium optimization studies (Figures 1.1, 1.2, and 1.5), and also the elimination of the futile NH_4^+ uptake transport system of AmtB (Figure 1.7), to determine maximum rates of NH_4^+ accumulation that could be achieved based on a culmination of all of these approaches. The results indicate a 2.7-fold increase in the NH_4^+ accumulated per hour (Figure 1.7 versus Figure 1.4), accumulating NH_4^+ at nearly 1 mM per hour under these conditions. In addition to the elevated levels of NH_4^+, OD_{600} values also increased, indicating that improvements were enabling a higher density of cells. Further improvements might be possible if oxygen delivery could be improved further without resulting in negative processes that result in aggregation, as was found when adding baffles to the culture.

In summary, this study provided insight into the limiting factors that *A. vinelandii* may face when de-regulated for BNF. These limitations showed that when amended, NH_4^+ levels achieved by this strain could be improved. Oxygen availability, temperature, sucrose concentration, ammonium re-uptake, and the amount of cofactor-related elements present in the media play key roles in maintaining this impressive phenotype.

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Chapter 2

Efforts toward optimization of aerobic biohydrogen production reveal details of secondary regulation of biological nitrogen fixation by nitrogenous compounds in *Azotobacter vinelandii*

Abstract

Biological nitrogen fixation (BNF) through the enzyme nitrogenase is performed by a unique class of organisms known as diazotrophs. One interesting facet of BNF is that in addition to ammonium (NH_4^+), it also produces molecular hydrogen (H_2) as a requisite by-product of the BNF reaction. In the absence of N_2 substrate, or under conditions that limit access of N_2 to the enzyme through modifications of amino acids near the active site, nitrogenase activity can be redirected toward a role as a dedicated hydrogenase, producing only H_2 gas. In free living diazotrophs, nitrogenases are tightly regulated to minimize BNF to meet only the growth requirements of the cell, and are often accompanied by uptake hydrogenases that oxidize the H_2 by-product to recover the electrons from this product. The wild-type strain of *Azotobacter vinelandii* performs all of the tasks described above to minimize losses of H_2 while also growing as an obligate aerobe. Individual alterations to *A. vinelandii* have been demonstrated that disrupt key aspects of BNF, thereby diverting resources and energy toward the production of H_2. In this work, we have combined three approaches to improve biological H_2 production by nitrogenase in *A. vinelandii*. In addition, the resulting H_2 producing strain was further utilized as a surrogate to study secondary, post-transcriptional regulation of BNF by several key nitrogen containing metabolites. The improvement in yields of H_2 that were achieved through various combinations of these three approaches were compared and are presented along with the insights into inhibition of BNF by several nitrogenous compounds that are common in various waste streams. The findings shed new light on which nitrogen compounds specifically hinder BNF through this secondary inhibition, and provide essential details to inform future biosynthetic approaches to yield nitrogen products that do not inadvertently inhibit BNF.

Introduction

Hydrogen gas (H_2) could augment fossil fuel usage due to its carbon neutral combustion and ability to generate electricity in fuel cell applications. Currently, H_2 is primarily produced from non-renewable sources such as fossil fuel reserves of natural gas (52). Reducing fossil fuel usage and mitigating climate change make the presently used thermochemical processes less desirable than biological ones in which H_2 is generated from waste streams that feed H_2 producing organisms or utilize photosynthetic species (53-55). Biologically generated H_2 (biohydrogen) provides an eco-friendly and renewable method with which to take advantage of these waste streams, but the processes yielding biohydrogen suffer from low productivity and high complexity which have thus far prevented them from being broadly adopted by industry (29, 56). Therefore, efforts to provide novel biohydrogen production routes are actively being pursued by laboratories across the globe.

There are currently two primary routes to biohydrogen production. The first is light-dependent and relies on a photosynthetic species such as an alga or cyanobacterium (46, 53-57). The second is light-independent (52, 58, 59), and occurs in many anaerobic environments, including in most marshland sediments. Both of these routes use either a nitrogenase or hydrogenase to generate H_2. The general biological nitrogen fixation (BNF) reaction catalyzed by the enzyme nitrogenase produces H_2 as a by-product when dinitrogen (N_2) in the atmosphere is reduced into ammonia (NH_3) as shown in Equation 1 (5);

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16(ADP + P_i) \quad \text{(Equation 1)}$$

Azotobacter vinelandii is a free-living, nitrogen-fixing bacterium (diazotroph). It is both a strict aerobe and has been studied for decades as a model organism for BNF, due to its ability to be easily genetically modified in addition to a fully sequenced and annotated genome (8). *A. vinelandii* naturally evolves very low levels of H_2 during BNF, as any H_2 produced is generally recycled by the uptake hydrogenase (Figure 2.1). Hydrogenase catalyzes the reversible oxidation of H_2 gas to recapture the electrons consumed by nitrogenase to reduce the energetic costs of BNF through Equation 2;

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad \text{(Equation 2)}$$

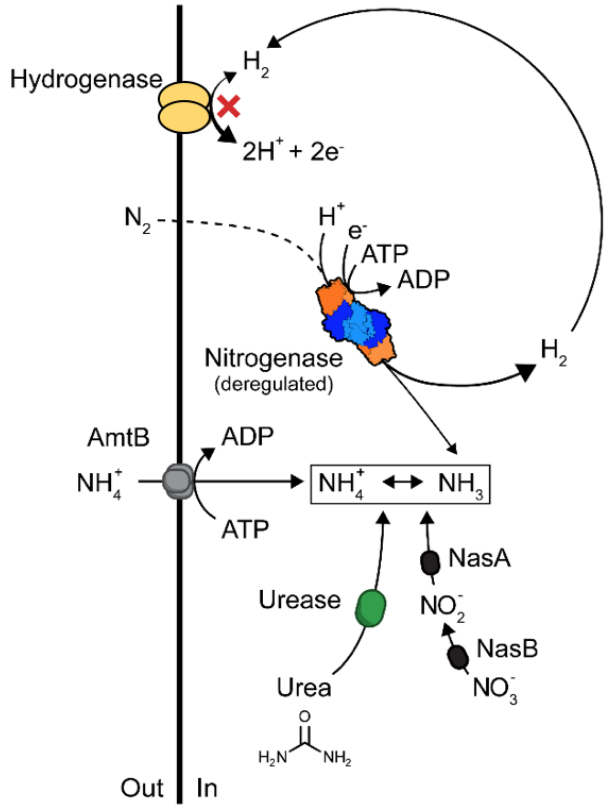

Figure 2.1: Simplified schematic showing select metabolic routes to hydrogen (H_2) and ammonia (NH_3) production in Azotobacter vinelandii. The diagram depicts strain AZBB275 which has a variant form of the Mo-dependent nitrogenase which is limited in its ability to fix dinitrogen into NH_3 (dashed line) but not limited in its proton reduction (larger arrow). This strain *is* also unable to consume H_2 as both hydrogenases have been knocked out (red x); only the membrane-bound hydrogenase is shown as it is *believed* to be the primary H_2 consumer associated with nitrogenase.

Many of the current approaches to produce biohydrogen only use hydrogenases (46, 56, 59, 60). However, this approach is limited to some extent by the reversible nature of the hydrogenase reaction (Equation 2). The H_2 by-product of nitrogenase is the result of a unidirectional reaction (Equation 1), which cannot be driven backwards to any great extent (20, 61). Therefore, in the absence of a hydrogenase, any H_2 produced by *A. vinelandii* would become a terminal product, assuming there are no other biological processes that would utilize this H_2 within the cell.

A. vinelandii is genetically tractable, and can be modified to increase its H_2 production. However, the full potential of the biohydrogen production scheme has not been adequately explored in *A. vinelandii*, although individual approaches that increase nitrogenase-driven H_2 production have been independently reported by employing both *in vivo* and *in vitro* methods (29, 31, 62). One such approach was to knock out the uptake hydrogenase genes in *A. vinelandii*, which results in H_2 gas production as the organism is no longer able to oxidize the H_2 by-product of BNF (6, 29). Another is to make certain amino acid substitutions near the active site of the FeMo-cofactor in the NifD subunit (α-subunit) of nitrogenase, resulting in a variant form of the enzyme which is deficient in its ability to reduce N_2, but unaffected in its ability to reduce protons to H_2 (31, 54). One specific amino acid substitution that shifts the selectivity of the nitrogenase active site is the modification to the *nifD* gene at the position of valine 70 ($nifD^{V70I}$) of the Mo-dependent nitrogenase, resulting in a smaller active site that no longer open enough to reduce N_2, but is still capable of reducing H^+ into H_2 (31).

The final approach to increase the levels of H_2 is by deregulating the expression of nitrogenase in *A. vinelandii*, which can be accomplished by modifying the regulatory operon *nifLA* in *A. vinelandii*, where NifA acts as an activator for nitrogenase expression and NifL acts as an anti-activator. This approach deletes *nifL* from the genome and was first reported in 1992, but recently reconstructed in our laboratory (10-12). The resulting strain yields considerable amounts of ammonium (NH_4^+) into the spent medium, and maintains elevated levels of nitrogenase expression during both the exponential and stationary phases of growth, compared to wild-type *A. vinelandii* which typically dramatically decreases nitrogenase expression during stationary phase growth (6). This indicated that a deregulated strain would continue to drive the activity of nitrogenase, even when substantial amounts of NH_4^+ have accumulated in the medium and cultures had reached stationary phase. We anticipated that by incorporating this genomic modification which deregulates the nitrogenase, along with the inability to recycle H_2 and the inclusion of a modification to nitrogenase that drives substrate preference away from N_2 and toward H^+ reduction, we might construct a strain that approaches the full potential of H_2 production by *A. vinelandii*.

Since H_2 is a by-product of the BNF reaction, it could also act as a proxy of nitrogenase activity if the reactions that recycled H_2 were removed. Generally, enzymatic characterization of nitrogenase is accomplished with either *in vitro* assays using purified enzyme or *in vivo* assays where live cells are subjected to the alternative substrate, acetylene. *In vitro* assays using pure enzymes are the optimal method of measuring nitrogenase activity, as this removes any effects of the regulatory system which modulates nitrogenase activity and expression. Biotechnological applications aimed at exploiting nitrogenase as it exists within an organism would require robust whole-cell assays. *In vivo* assays using acetylene are hindered by low nitrogenase levels and activities, and require that acetylene, a substrate for the enzyme and also an inhibitor of BNF, always be present. This prevents testing of environmental conditions where no inhibitor is desired or in studies aimed to isolate the effect of a single compound on the internal functions of the cell.

Our goals in this study were twofold. First, we sought to optimize the internal pathways of *A. vinelandii* through genome engineering to improve H_2 production by the molybdenum-based nitrogenase. Second, we aimed to demonstrate the utility of our optimized strain to serve as a tool for monitoring whole-cell nitrogenase activity in response to additional nitrogenous compounds found in waste streams (which may be used as a feedstock for this strain) or as potential metabolites in the cell, including NH_3, the natural product of the nitrogenase enzyme. Our results reveal a unique secondary inhibition profile for various nitrogenous compounds that appears to be novel and acting outside of primary regulation. Once we uncovered this secondary inhibition of BNF, we further investigated this by eliminating additional pathways which are responsible for metabolizing these compounds to identify which specific chemical species were inhibiting BNF, and which might be benign if left unaltered. These results provide strong evidence that NH_4^+ and nitrite deregulate BNF, while nitrate and urea have little effect on this secondary inhibition. A summary of these results and the implications on BNF and potential biosynthetic approaches to increase yields of BNF toward novel nitrogen compounds is presented.

Methods

Reagents, bacteria and media. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). *Azotobacter vinelandii* DJ (ATCC BAA-1303) was obtained from Dennis Dean (Virginia Tech) and cultured on Burk's (B) medium (26). *A. vinelandii* strains (Table 2.1) were grown aerobically at 30°C using B medium, which was prepared as described previously and adjusted to pH 7.5 (26). For H_2 assays, strains were grown in sterile filtered B medium supplemented with 20 mM MOPs buffer. Kanamycin was excluded from the assays, but included on plates used to maintain specific strains. Strains containing the *nifD*^{V70I} amino acid substitution were grown on B medium agar plates supplemented with urea (10 mM) or nitrate (10 mM). Ammonium was provided as ammonium sulfate.

Construction of A. vinelandii strains. All plasmids were constructed and maintained within *Escherichia coli* JM109 which was obtained from New England Biolabs (Ipswhich, MA). The construction of strains AZBB163 and DJ1373 and methods for *A. vinelandii* genome editing have been described previously (11, 27, 26). A complete list of the strains, plasmids and primers used are listed in Tables 2.1, 2.2 and 2.3 respectively.

Table 2.1. Key strains constructed and utilized in this study

Strain	Genetic Features	Plasmid Used	Parent strain
DJ*	Wild-type *A. vinelandii* with diminished alginate production resulting in ease of transformation.	None	(8)
DJ1373*	*nifD*::*nifD*^{V70I}		(31)
AZBB163*	*nifL*::kan^{R}-(pPCRNH3-43), grown in nitrogen limited medium, and mutated through directed evolution to a *nif*+ phenotype which produced high levels of extracellular ammonia.	pPCRNH3-43, spontaneous mutation	AZBB150
AZBB198	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*::*pyrF*-kan^{R}	pPCRHYDK9	AZBB105 (27)
AZBB212	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*	pPCRHYDK7	AZBB198
AZBB219	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*, *hoxKGZMLOQRTV*::*pyrF*-kan^{R}.	pPCRHYDK10	AZBB212
AZBB233	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*	pPCRHYDK8	AZBB219
AZBB245	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifD::pyrF*-kan^{R}	pPCRNIF33	AZBB233
AZBB261*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*,	pPCRPYRF1	AZBB233
AZBB263	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*pyrF*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifD*::*nifD*^{V70I},	pPCRNIF32	AZBB245
AZBB269*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifL*::kan^{R}-pPCRNH3-44	pPCRNH3-44	AZBB261
AZBB271*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifD*::*nifD*^{V70I}	pPCRPYRF1	AZBB263
AZBB275*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifD*::*nifD*^{V70I}, *nifL*::kan^{R}-pPCRNH3-44	pPCRNH3-44	AZBB271
AZBB312*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifD*::*nifD*^{V70I}, *nifL*::kan^{R}-pPCRNH3-44, Δ*ureABC*::strep^{R}	pPCRURE3	AZBB275
AZBB330*	Δ*vnfHDGK*, Δ*anfHDGK*, Δ*hoxN*$_1$*IN*$_2$*YHW*, Δ*hoxKGZMLOQRTV*, *nifD*::*nifD*^{V70I}, *nifL*::kan^{R}-pPCRNH3-44, Δ*nasAB*::strep^{R}	pPCRNRK18	AZBB275

N$_1$ = *Avin_04360* and *N*$_2$ = *Avin_04380*; strep^{R}, streptomycin/spectinomycin resistance; kan^{R}, kanamycin resistance.
Strains marked with (*) are completed strains used in this study

Table 2.2. Primers used in this study

Primer	Sequence (5' to 3')	Purpose
BBP950	GAGCACACCC ATCACGGTCA GAG	*nifLA* modification confirmation
BBP982	GAAGGGCAGC AGCAGGTAGA GG	*ureABC* deletion confirmation
BBP983	CAGCAGTTCGCGAAGACTGTCGAAG	*ureABC* deletion confirmation
BBP1038	GACACGGATC CCTACGAAGA CGATCTGGAC TTCGTATTCT A	*nifHDK* cloning
BBP1100	GACACTCTAG AAGTAGCGAG CAATGCCTTC GAACATGTTG TC	*nifHDK* cloning
BBP1207	GAGTGGATCC GACAGAGCCA ATCTGCCCTG TC	*nifHDK* deletion
BBP1208	GACAGGATCC AATCGTACGC AACGTCCGCT G	*nifHDK* deletion
BBP1292	GTTGTAGCAA TTACAACAGT CGGAGTAGG	*nifHDK* deletion confirmation
BPP1293	GTAGCTGTTG CCGATCATGA AGTC	*nifHDK* deletion confirmation
BBP1322	GATCTCCATC GACTCGATCT TGTCCAGGGT GAAC	*nifLA* modification confirmation
BBP1335	GTAAGTCCAC CACTACTCA GAACCTGGTG GCAG	*nifLA* modification confirmation
BBP1336	GAGTCGGTCA TCATGTCGAC CAGACG	*nifLA* modification confirmation
BBP1883	CGATCACCGA GCCAAACACC ACTATCAG	*pyrF* deletion confirmation
BBP1884	CGCTGGTCGT TGCACCAACT CGGATGAG	*pyrF* deletion confirmation
BBP1237	GACTAAGCTT CTCGACTTCG GCCTGGCCTA CTG	*nasAB* cloning
BBP2376	NNNTCTAGAC AGTTCAGGCT GCTGTCGTCC TGGGTGTG	*nasAB* cloning
BBP2377	NNNAGATCTT CTCCGTTACG GAAAGCGG	*nasAB* deletion
BBP2378	NNNAGATCTG AATCCGGGAC GGAAGAGCGC ACAAC	*nasAB* deletion
BBP1397	CTGTTGCTGG AGGAATGGTT CCTGCGTC	*nasAB* deletion confirmation
BBP2394	CGTGATGTTC TGGTCAACGA GTATGGC	*nasAB* deletion confirmation
BBP2395	GTCTGCCAGG ACTTCGCCCA CCTGATGCTC	*hox*N_1*IN*$_2$*YHW* deletion confirmation
BBP2396	CAGCGACGGC GTGGTCAGGT ACAGGTC	*hox*N_1*IN*$_2$*YHW* deletion confirmation
BBP2397	GTCGTCCAGC ACGGTCAGTG GATGACAG	*hoxKGZMLOQRTV* deletion confirmation
BBP2398	GCTGACGCTC GATCCAGGCC AGCCACTC	*hoxKGZMLOQRTV* deletion confirmation
BBP2449	CGGTTCCAAA GGCATAGTCT GGGGCCCCAT C	V70I change in *nifD*
BBP2450	GATGGGGCCC CAGACTATGC CTTTGGAACC G	V70I change in *nifD*

N_1 = Avin_04360 and N_2 = Avin_04380

Table 2.3. Plasmids and plasmid derivatives used in this study

Plasmid[a]	Gene(s) Cloned or Plasmids Manipulated[b]	Parent Vector	Source
pBB053	Removed NdeI site from pUC19 by silent mutation	pUC19	(28)
pPCRHYDK7	Combined two regions flanking *hoxN_1IN$_2$YHW* from pPCRHYDK3 and pPCRHYDK4 into new vector	pPCRHYDK3	(6)
pPCRHYDK8	Combined two regions flanking *hoxKGZMLOQRTV* from pPCRHYDK5 and pPCRHYDK6 into new vector	pPCRHYDK5	(6)
pPCRHYDK9	Insertion of kan^R and *pyrF* cassette between the flanking regions of *hoxN_1IN$_2$YHW* for deletion via *pyrF* counter selection.	pPCRHYDK7	This study
pPCRHYDK10	Insertion of kan^R and *pyrF* cassette between the flanking regions of *hoxKGZMLOQRTV* for deletion via *pyrF* counter selection.	pPCRHYDK8	This study
pPCRKAN15	kan^R and *pyrF* cassette used for insertion for *pyrF* and counter selection.	pPCRKAN11	(27)
pPCRNIF10	Cloned *nifD* from *A. vinelandii* and inserted into host vector and removed unwanted restriction sites for further manipulation.	pBB053	
pPCRNIF11	Removed segment of *nifD* and inserted new BamHI site.	pPCRNIF10	This study
pPCRNIF32	Site-specific mutagenesis to convert valine-70 to isoleucine in NifD.	pPCRNIF10	This study
pPCRNIF33	Insertion of kan^R and *pyrF* cassette between the flanking regions of *nifD* in *A. vinelandii* to be used for markerless deletion of *nifD*.	pPCRNIF11	This study
pPCRNH3-44*	Incorporated C to T mutation into kanamycin cassette region to create Nif$^+$ phenotypes with high ammonium production	pPCRNH3-43	(6, 11)
pPCRNRK10	Cloned *nasAB* and flanking regions from *A. vinelandii* and inserted into pBB053.	pBB053	This study
pPCRNRK11	Removal of *nasAB* from parent vector, leaving flanking regions for insertion of antibiotic cassette.	pPCRNRK10	This study
pPCRNRK18*	Insertion of strep^R cassette between flanking regions of *nasAB*, to be used to delete *nasAB* in *A. vinelandii*.	pPCRNRK11-2	This study
pPCRPYRF1*	Cloned the *pyrF* gene region from *A. vinelandii* and insertion into pBB053.	pBB053	(27)
pPCRURE3*	Insertion of strep^R cassette between flanking regions of urease in *A. vinelandii* for use in creating strains with deletion.	pPCRURE2	(11, 28)

[a] The sequences of all plasmids in this study are available upon request. Plasmids indicated by an asterisk (*) are completed vectors that were used to transform *A. vinelandii*

b strep^R, streptomycin resistance; kan^R, kanamycin resistance;

c N_1 = Avin_04360 and N_2 = Avin_04380

Protein quantification. To quantify the total amount of starting protein, isolated cells were suspended in one mL of water and sonicated for 50 seconds (Misonix LX-2000, Qsonica, Newtown, CT) inside of a 1.5 mL tube. Cell lysate was then added to 1.0 mL of Coomassie Plus (Bradford) assay reagent (Pierce, Rockford, IL), mixed by inverting the cuvette several times and incubated at room temperature for 10 minutes. Samples were read at a wavelength of 595 nm (Varian Inc., Palo Alto, CA). Samples were compared to a standard curve prepared using Bovine Serum Albumin as a standard (Pierce, Rockford, IL).

Hydrogen measurements. Hydrogen gas (H_2) was measured by transferring a specific amount of cells scraped from a two-day old plate to 3 mLs of medium in a 20-mL serum vial such that the starting OD_{600} was 1.5. The serum vials were then capped with stoppers, sealed and pre-incubated for two hours at 30°C, 180 RPM and a 45° angle. This pre-incubation was incorporated to assure that cells had adapted to liquid culture conditions prior to initiating any measurements. After the two hours, the culture head space was flushed with fresh air, and the serum bottles were resealed. At this point, 250 µL was drawn from the headspace and analyzed. The sample was then allowed to incubate for another two hours under the same conditions. Sampling was done during the second incubation period at regular intervals depending on the assay. Gas composition of the headspace was determined using a gas chromatograph equipped with a thermal conductivity detector (GC-8A, Shimadzu Scientific) with argon as the carrier gas and a molecular sieve 5A column, similar to what has been described previously (63, 64). H_2 consumption was quantified by the same method, but a starting OD_{600} of 0.375 was used and 250 µL of H_2 at atmospheric pressure was added after thoroughly venting the culture. This lower OD_{600} was selected for accurate measurement of H_2 consumption, as control experiments revealed that gas transport and solubility could limit activity at higher cell concentrations of cells.

Results

Nitrogenase driven H_2 production. Three modifications were incorporated into the *A. vinelandii* genome to increase the production of H_2 by this model bacterium: a modification to *nifL* that deregulates the expression of nitrogenase (10-12), incorporation of a mutation near the active site of nitrogenase that results in a preference of the enzyme for H^+ reduction over N_2 reduction (31), and deletion of the uptake hydrogenase complex that recycles the H_2 by-product of the nitrogenase reaction. Various combinations of these three modifications were constructed and are shown in Figure 2.2 in order of increasing H_2 production (blue bars). The strain AZBB275 incorporating all three modifications had the highest H_2 production (5.6 μmol h^{-1} (mg protein)$^{-1}$), which was 28 times greater than that of the wild-type strain DJ (0.2 μmol h^{-1} (mg protein)$^{-1}$). The largest effect by a single component modification was seen for the hydrogenase double knock-out strain, AZBB261. The variant form of the molybdenum-dependent nitrogenase containing the *nifD*^{V70I} amino acid substitution (DJ1373) resulted in an increase in H_2 production to around 0.7 μmol h^{-1} (mg protein) $^{-1}$, indicating that this mutant alone results in sufficient H_2 production to overwhelm the capability of the uptake hydrogenase to recycle the H_2. The majority of these strains were constructed from a parent strain devoid of either the vanadium-dependent or iron-only nitrogenases (Δ*vnfHDGK* and Δ*anfHDGK*, respectively (27)) to assure that the results measured are solely attributed to the molybdenum-dependent nitrogenase of *A. vinelandii*, although these other two nitrogenase systems would not be expected to be highly expressed under the growth conditions employed in this study, which included molybdenum in the medium (6). Additionally, we also deleted the second hydrogenase gene cluster, which is not generally associated with hydrogen uptake (6, 30), to further assure that no alternative pathways to consume hydrogen are present in the target strain.

Nitrogenase inhibition by NH_4^+. The effect of elevated levels of ammonium (NH_4^+) in the medium was also probed in this study. The inclusion of 5 mM NH_4^+ in the growth medium reduced the levels of H_2 produced by all strains (Figure 2.2, grey bars). This concentration of NH_4^+ was chosen as it was found to result in strong inhibition in the hydrogenase deletion strain (AZBB261), though it should be pointed out that these levels of NH_4^+ are much higher than anything that would accumulate in any of the strains

except AZBB269 containing the modification to the *nifL* region, and even AZBB269 does not accumulate these levels of NH_4^+ during the short timeframe of the assays employed here. In the final strain AZBB275, there was a 5-fold reduction when compared to growth in nitrogen free medium. Both of the strains (AZBB269 and AZBB275) containing the *nifL*::kan^R modification retained the ability to produce H_2 at ~1 µmol h^{-1} (mg protein) $^{-1}$. Near complete repression of nitrogenase activity by 5 mM NH_4^+ was seen in almost all of the strains lacking the *nifL* modification (Figure 2.2, all but AZBB269 and AZBB275). AZBB163, which contains the *nifLA* modification alone, did not product much hydrogen, so the apparent inhibition by NH_4^+ was enough to nullify this already small amount. Strain AZBB275, which contains the *nifD*^{V70I} amino acid substitution, is essentially unable to fix nitrogen under the conditions employed in these assays or even under typical growth conditions, which would limit any background inhibition by NH_4^+ in this optimized strain. One caveat of the *nifD*^{V70I} modification is that it makes these strains dependent on an external source of nitrogen to attain similar growth rates to the other strains (Table 2.4).

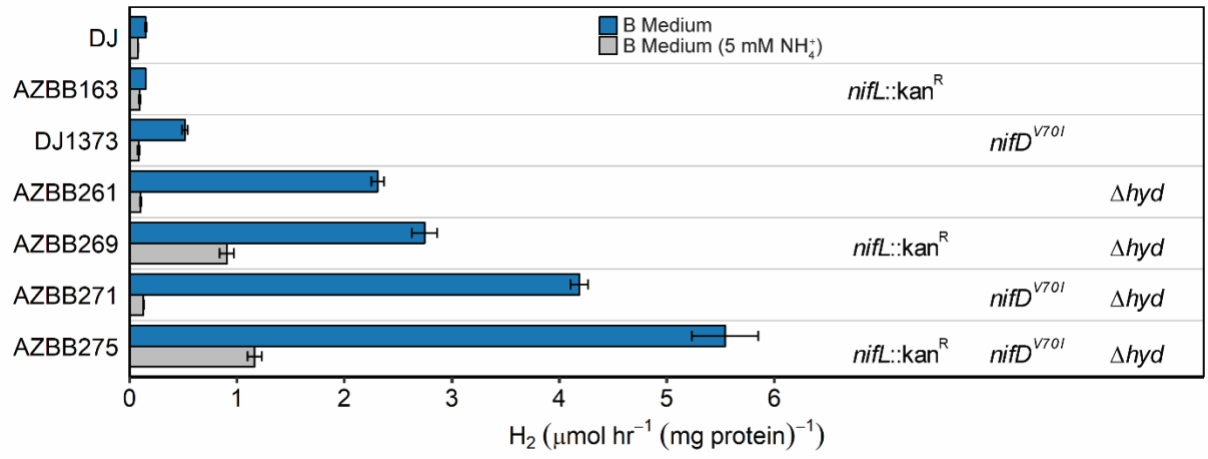

Figure 2.2: Hydrogen produced by wild-type and mutant strains of A. vinelandii in the absence (blue bars) or presence (grey bars) of 5 mM ammonium (NH_4^+). Relevant genetic features of each strain are listed on the right. Strains with nifD^{V70I} result in a nif$^-$ phenotype. Strains designated with Δhyd are lacking both the soluble (Avin_04360 – Avin_04410) and membrane-bound (Avin_50500 – Avin_50590) hydrogenases. Data represents averages, while error bars represent standard deviation (N = 3).

Table 2.4: Doubling time of strains used in this study.

Strain	*B Medium*	*B Medium (10 mM Urea)*
	Doubling time (hours)	Doubling time (hours)
AV Trans	3.27 ± 0.15	2.35 ± 0.06
AZBB163	4.16 ± 0.09	2.78 ± 0.06
AZBB269	4.35 ± 0.25	3.22 ± 0.31
AZBB275	110.66 ± 4.94	3.29 ± 0.12

Continuous production of H_2. To determine if the rates of H_2 production were changing during the 2-hour assay, levels of H_2 and oxygen (O_2) were measured in 15 minute increments. Linear H_2 production was observed throughout the 2-hour duration of the assay (Figure 2.3), indicating that rising levels of H_2 were not inhibiting the further production at these concentrations. This was further correlated with a linear decrease in O_2 reaching a final concentration indicating 33% consumption of O_2. This was in agreement with a preliminary experiment indicating that cells required about 2 hours following introduction into the liquid culture to achieve maximal exponential growth and hydrogen production levels. This 2 hour pre-incubation time was employed throughout all of these experiments.

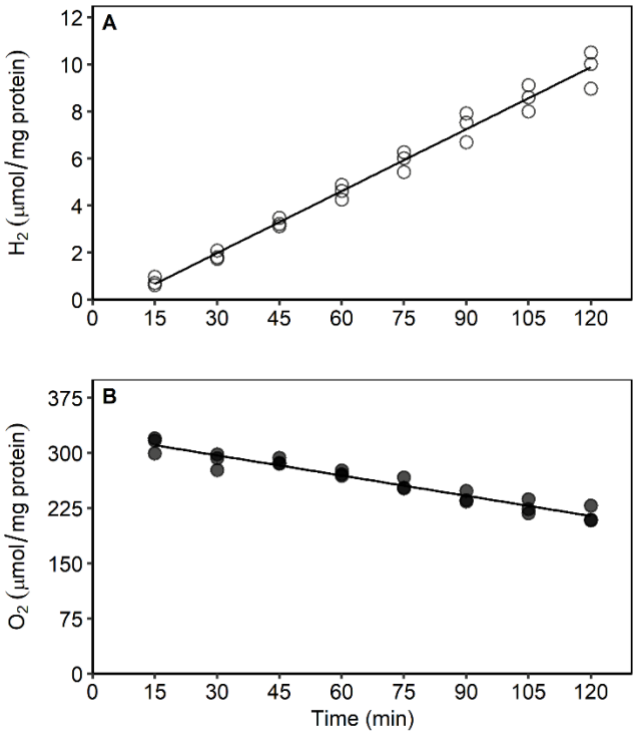

Figure 2.3: H_2 production and O_2 consumption by A. vinelandii strain AZBB275 over time. (A) Hydrogen and associated (B) oxygen levels reported as µmol per mg starting protein (N = 3, linear least squares regression).

Production versus consumption rates of H_2. Strains AZBB275 and AZBB163 were further tested for the production or consumption of H_2 by *A. vinelandii* to compare and contrast these two competing reactions (Figure 2.4). A volume equivalent to 9 µmol of H_2 (slightly greater than the amount produced during two hours by AZBB275 in this experiment) was added to three of the four cultures. Strain AZBB275 showed linear H_2 production up to 16 µmol of H_2 when initially started with this spiked amount of H_2. Supplementing AZBB275 with exogenous NH_4^+ (20 mM) reduced the rate of production, but not entirely, similar to what was found in Figure 2.2. Strain AZBB163, which still contained both of the hydrogenase complexes, consumed H_2 at a rate greater than its production capabilities, resulting in a continuous decrease in the H_2 that was spiked into this sample. Rates of H_2 production by AZBB275 were similar in the samples with or without the initial quantity of spiked H_2, further confirming that the H_2 produced was not limiting further production of H_2 through any noticeable inhibition.

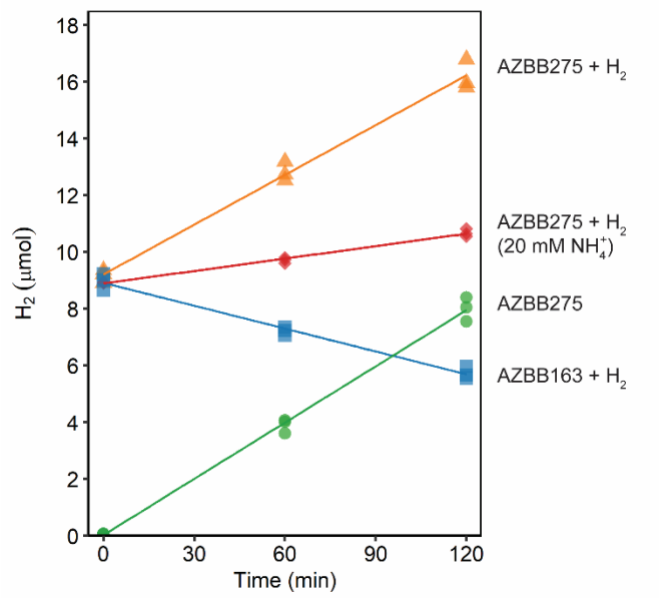

Figure 2.4: H_2 produced by A. vinelandii strains AZBB275 and AZBB163 when cultured under conditions with or without exogenously provided ammonium and exogenously provided H_2. AZBB163 was introduced at an OD_{600} of 0.375 while all AZBB275 samples were introduced at an OD_{600} of 1.5. Samples to which 250 µL H_2 was added are noted (+ H_2). (N = 3, linear least squares regression).

Additional nitrogenous compounds as inhibitors of nitrogenase. NH_4^+ added to the medium inhibited H_2 production by AZBB275 (Figures 2.2 and 2.4). To determine the potential of other N compounds to inhibit H_2 production, AZBB275 was screened with urea, nitrite (NO_2^-) and nitrate (NO_3^-). AZBB275 is suitable for this analysis because the *nifD*^{V70I} amino acid substitution results in a very limited ability to fix N_2, so that NH_4^+ levels accumulating within the cell would be negligible. Nitrogenase activity was repressed by all three of these N compounds in addition to NH_4^+ (Figure 2.5); ~80% with 10 mM NH_4^+, ~70% with 3 mM NO_2^-, ~40% with 10 mM NO_3^- and ~70% with 10 mM urea. As the rates of inhibition by NH_4^+ and NO_2^- were greater than what was found for urea and NO_3^-, we speculated that it might not be urea or NO_3^-, but instead the products of further enzymatic activities within the cell that were resulting in their inhibition. Repression by urea was removed in strain AZBB312, which is based on strain AZBB275, but also contains a further disruption of the gene coding for the enzyme urease (*ureABC*),

which would hydrolyze urea into carbon dioxide and NH_4^+ (Figure 2.1). Strain AZBB330, which is also based on AZBB275, contains disruptions to the genes coding for both nitrate reductase and nitrite reductase (*nasAB*) (Figure 2.1), eliminating further reduction of NO_3^- to NO_2^- or NO_2^- to NH_4^+, rendering the NO_3^- a terminal compound within the cell. Inhibition by NO_2^- remained as was found for AZBB275 while NO_3^- inhibition was removed in the knock-out strain AZBB330. The inability to further reduce NO_2^- to NH_4^+ was also confirmed for AZBB330. Concentration curves for NH_4^+ and NO_2^- (Fig. 6) in strains AZBB275 and AZBB330 show sensitivity to both compounds that increased in a concentration dependent manner.

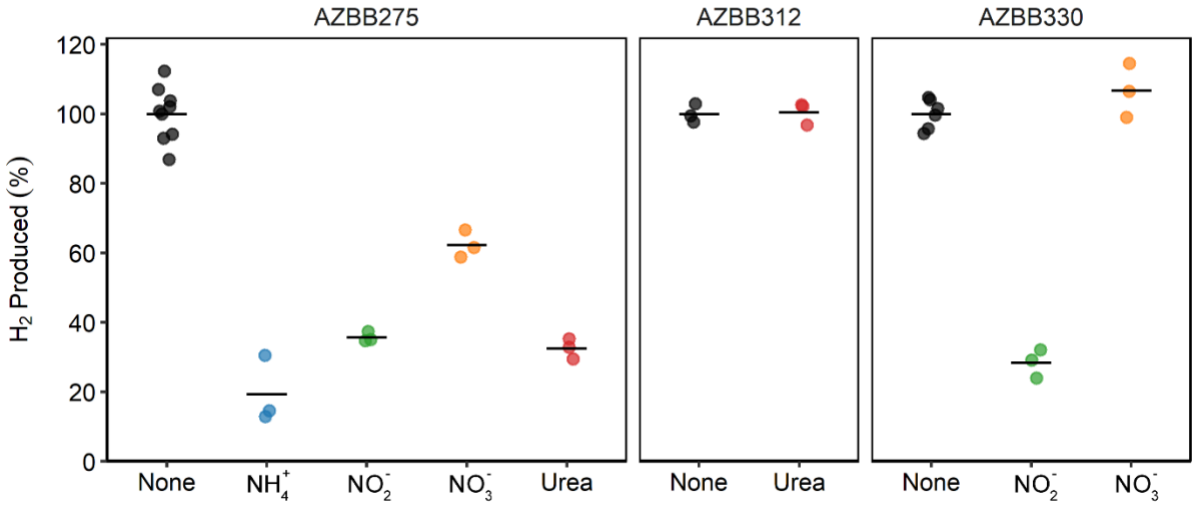

Figure 2.5: Effect of different nitrogen compounds on the amount of H_2 produced by A. vinelandii AZBB275 and the two mutant strains AZBB312 (AZBB275 + ΔureABC) and AZBB330 (AZBB275 + ΔnasAB). Strains were incubated with medium that was not supplemented with any nitrogenous compounds, 10 mM NH_4^+, 3 mM nitrite (NO_2^-), 10 mM nitrate (NO_3^-) or 10 mM urea (CH_4N_2O). ($N \geq 3$, mean)

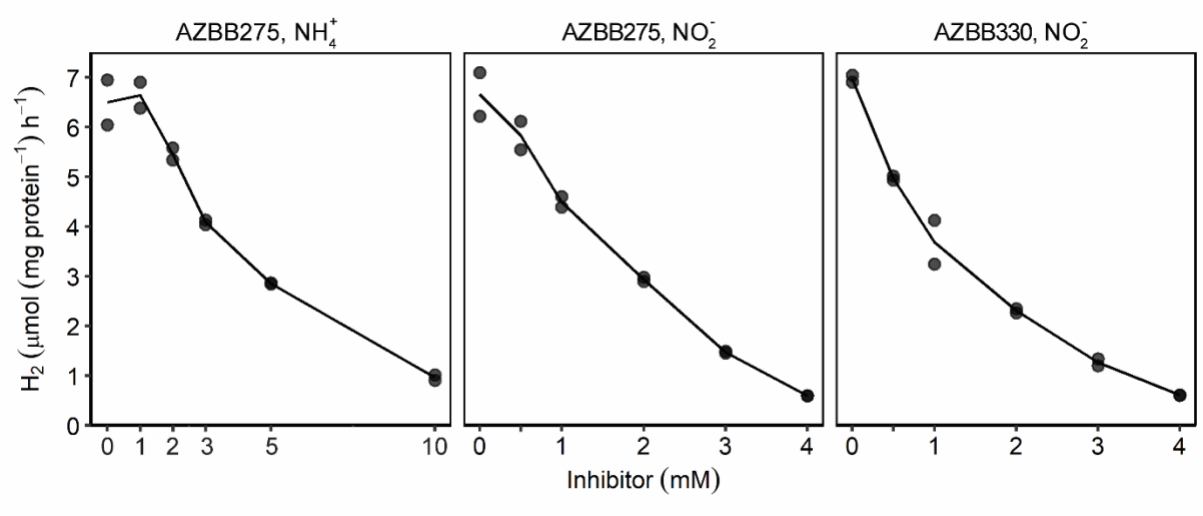

Figure 2.6: Effect of the concentration of NH_4^+ and NO_2^- on H_2 production in A. vinelandii strains AZBB275 and AZBB330 (AZBB275 + ΔnasAB). (A) AZBB275 with NH_4^+, (B) AZBB275 with NO_2^-, (C) AZBB330 with NO_2^-. (N = 2, running average)

Full cell assay susceptibility to pH effects. The effect of pH on H_2 production in *in vitro* assays using isolated nitrogenase (activity measured in an argon atmosphere) was studied previously (65). However, the strains constructed here allow for *in vivo* measurement of the effect of the pH of the growth medium and the ability of the cells to produce nitrogenase driven H_2. H_2 production increased with increasing pH over the range of 5.5 – 9.0. All other experiments described in this study were done in medium adjusted to pH 7.5, which was selected because it is close to the starting pH of typical B medium. Therefore, the H_2 values presented in Figure 2.7 were normalized to the mean value obtained for the pH 7.5 samples and converted to percentages. *A. vinelandii* struggled to grow at pH levels lower than 6 and showed minimal H_2 production at low pH. Maximum H_2 production (135%) plateaued at pH values of 8.5 and 9, indicating that mild alkaline conditions favor nitrogenase activity *in vivo*.

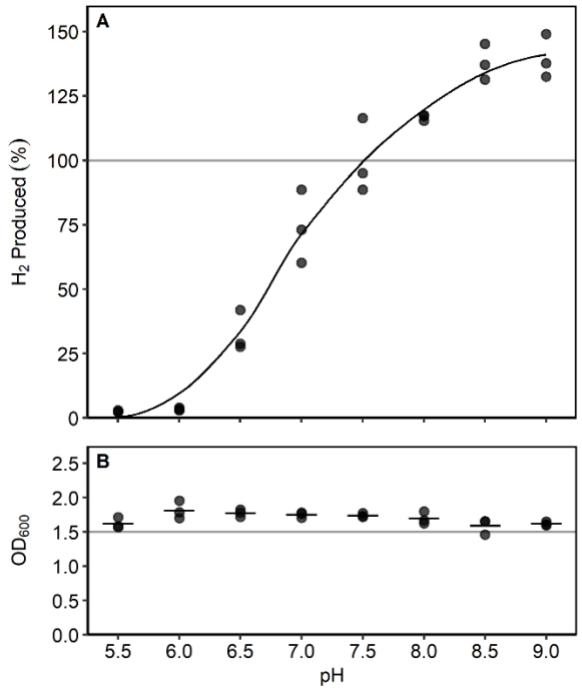

Figure 2.7: Effect of pH on H_2 production and OD_{600} in A. vinelandii strain AZBB275. (A) H_2 produced and associated (B) OD_{600} measurements obtained at the end of the 2 hour assay. B medium was adjusted to pH 7.5 in all other experiments so all values in this figure were normalized to the mean of the pH 7.5 samples. (N = 3, LOESS regression and mean)

Discussion

The initial goal of this study was to determine the potential to produce hydrogen gas (H_2) via nitrogenase when the path to H_2 was optimized in *A. vinelandii* using a combination of three different strategies. We constructed and measured each individual modification to yield increasing levels of H_2 in isolated strains both individually, and in combination (Figure 2.2). Prior to these experiments, it was uncertain whether the effects of these combined modifications in *A. vinelandii* would complement one another to increase H_2 yield, or result in a similar level of H_2 production to what was seen for the individual modifications (6, 10, 29, 31). By constructing and analyzing all three approaches alone and in combination, we were able to assess the independent contributions of each modification (Figure 2.2). Our final strain AZBB275 contains modifications resulting in the deletion of 28 genes, and mutations to two additional genes

to generate the central strain in these studies. H_2 is produced by a range of bacteria, often through the action of reversible hydrogenases, and generally as a result of anaerobic processes (52, 58, 59). *A. vinelandii* is unique from many bacteria, as it is an obligate aerobic nitrogen-fixing bacterium (diazotroph), and as such, it should be able to yield substantial H_2 through the mechanism of nitrogenase under aerobic conditions (8, 30).

Rey *et al.* pursued a similar approach to redirect metabolism in *Rhodopseudomonas palustris* toward biological hydrogen production, but required anaerobic conditions to accomplish this task (45). Fixen *et al.* built upon the work of Rey *et al.* to achieve elevated H_2 production and low levels of carbon dioxide and carbon monoxide reduction to shorter hydrocarbons by nitrogenase in *R. palustris* (66). Our modified *A. vinelandii* strain produced H_2 at rates greater than those obtained by Fixen *et al.*, who reported ~2 μmol h^{-1} (mg protein)$^{-1}$ (based on Figure 1B within reference (66)). Prior studies have also reported H_2 production in *A. vinelandii* based on disruptions of hydrogenase (6, 29, 30, 47), which would be comparable to what was obtained here for our hydrogenase disruption strain AZBB261. Our maximum yields of H_2 were approaching 7 μmol h^{-1} (mg protein)$^{-1}$ (Figures 2.2 and 2.7) in our strain containing all three modifications. The maximum production of H_2 using purified molybdenum-dependent nitrogenase through *in vitro* studies is approximately 2200 nmol min^{-1} (mg MoFe protein)$^{-1}$ (~132 μmol h^{-1} (mg protein)$^{-1}$) (31), indicating that at least ~ 5% of the protein in AZBB275 is active MoFe enzyme in the cells. Nitrogenase is a two component enzyme composed of the catalytic enzyme MoFe and the obligate reductase Fe protein. This high level of MoFe enzyme by convention indicates a similarly high level of Fe protein.

Masikawa *et al*. pursued a strategy in cyanobacteria including a deletion to the uptake hydrogenase and modifications near the active site of the molybdenum nitrogenase (54), and yielded results as high as ~24 μmol h^{-1} (mg chlorophyll *a*)$^{-1}$. Weyman *et al.* and Schütz *et al.* reported yields of 100-140 nmol h^{-1} (μg chlorophyll *a*)$^{-1}$ based on modifications resulting in disruption of the uptake hydrogenase and modification near the active site of the MoFe protein of nitrogenase, respectively (55, 67). Since each of these analyses is based on chlorophyll content, and not protein, it is difficult to make direct comparisons to our strains and yields. Bandyopadhyay *et al.* demonstrated yields of 3.5 μmol h^{-1} (mg protein)$^{-1}$ in *Cyanothece* sp. ATCC 51142 grown under anaerobic

conditions, though the levels under aerobic conditions were less than half of those rates (53). An accurate comparison of each of these strains would require the analysis to be performed and reported using the same benchmark, as either total protein or total cell mass, and would need to also consider levels of nitrogenase enzyme present and the source of energy to drive the process (heterotrophic versus photoautotrophic growth).

Our next goal in this work was to use strain AZBB275 as a surrogate to assay intracellular nitrogenase activity in response to physiological N compounds. The intent of this analysis was to determine if H_2 production, and by extension ammonium (NH_4^+) production (66), could be increased over current levels (29, 30, 47). It is well established that the presence of various nitrogen compounds within the growth medium will repress biological nitrogen fixation (BNF) in *A. vinelandii* (9, 68, 69), and that the specific disruption to the *nifL* gene, resulting in nitrogenase deregulation, is able to produce copious quantities of NH_4^+ (6, 10-12), which might further inhibit BNF. As our strains contain different combinations of these modifications, including the *nifL* disruption that dramatically derepresses nitrogenase expression, it was of interest to determine if the presence of NH_4^+ or different nitrogen compounds in the medium would continue to inhibit nitrogenase in this deregulated strain.

Repression of H_2 production by high levels (5 mM) of NH_4^+ in AZBB275 (Figure 2.2) demonstrates that BNF has yet to be fully deregulated, even in the high NH_4^+ producing strain AZBB163 that is proving to be a powerful tool to better understand global nitrogen regulation and response within the cell (6, 11). As AZBB163 can produce around 30 mM NH_4^+ (6, 12), and our results indicate that BNF is inhibited by levels of NH_4^+ as low as 3 mM (Figure 2.6), it is clear that secondary inhibition by the product is still limiting the potential yields that could be obtained by this strain. This suggests that there is a potential to improve yields further if the secondary repression mechanism in *A. vinelandii* could be identified and subsequently eliminated. In addition, this may imply that AZBB275, which contains the modification that results in a substrate preference toward H^+ reduction, would limit N_2 reduction thusly producing low to non-existent levels of NH_4^+, potentially avoiding this inhibition of nitrogenase activity by the cell's own produced NH_4^+ in more long-term studies.

In addition to the inhibition by NH_4^+, we also found inhibition by urea, NO_2^- and NO_3^-, though the urea inhibition could be eliminated if urease was disrupted and NO_3^- inhibition could be eliminated if nitrate reductase was disrupted, indicating that the primary inhibitory compounds were NH_4^+ and NO_2^-. Therefore, NH_4^+ inhibition might be overcome if fixed nitrogen could be directed toward terminal products of urea or NO_3^-. The nitrogenous compound NO_3^- is reported to repress BNF in *A. vinelandii* and *Cyanothece* sp. ATCC 51142 (35, 53, 70), but our results demonstrate that this inhibition in *A. vinelandii* is likely the result of NH_4^+ and NO_2^-, and not NO_3^-. Further optimization could be achieved by combining the disruption or deletion of nitrate reductase and urease to remove the inhibitive effects of NO_3^- and urea that may be present in nutrient rich waste streams which could be used as a potential feedstock for these strains. This also indicates that higher direct biological production of nitrogen compounds for biofertilizer applications could be achieved if products could be directed toward urea or nitrate in strains lacking urease or nitrate reductase, respectively.

Deregulation of BNF in these strains is accomplished by disrupting the *nifL* gene, which results in the constitutive expression of NifA in the absence of the repressor NifL(6, 10-12). As such, the data suggests that the diminished activity of nitrogenase observed in the presence of NH_4^+ is likely due to a post-translational modification that has yet to be adequately detailed or described in *A. vinelandii*. Other organisms use post-translational regulation through the actions of DraT and DraG (45, 71). These two proteins perform ADP-ribosylation of NifH in response to NH_4^+ (71). While comparative genomics has not revealed any homologs to DraT and DraG in *A. vinelandii*, a recent study proposed NifO as being similar in function to DraG when they adjusted the algorithm to better match different motifs (72). Based on the conclusions drawn from our study, identification of the gene(s) responsible for encoding potential secondary regulatory protein(s) could result in large improvements in nitrogenase activity, and further the potential for aerobic hydrogen or NH_4^+ production by *A. vinelandii*.

Being able to remove nitrogenase repression by NH_4^+ would allow for the use of this strain for the aerobic production of H_2 in N rich environments. If nitrogenase activity were insensitive to NH_4^+, the productivity of this strain and range of applications would

increase. Environments where external NH_4^+ was readily available would allow the strain to continue producing H_2 while not limiting its ability to use NH_4^+ for growth.

The pH dependency of H_2 production in active cultures was a curious phenomenon. Hydrogenases are well known to be pH dependent. However, strain AZBB275 no longer has either hydrogenase cluster. Because our strain lacks all known hydrogenase systems and both of the alternative nitrogenases (vanadium and iron only), it is assumed that all the H_2 produced in AZBB275 is a direct result of the molybdenum-dependent nitrogenase. Nitrogenase is also known to be pH sensitive with an optimum pH of 7.5 when tested *in vitro* (65). It is uncertain if the pH effect seen with AZBB275 is related to an effect on the nitrogenase within the cell, or if it has altered the overall metabolism to favor the production of H_2. Interestingly, our results indicated that the production of H_2 favors low availability of protons in the medium (Figure 2.7). Nitrogenase driven H_2 production has some advantages over fermentative and photosynthetic processes. *A. vinelandii*, as an aerobic, heterotrophic organism with no light requirement could potentially be modified to withstand fixed N sources. This makes it an interesting candidate for producing H_2 from various waste streams, which are often both nutrient rich and opaque. This study presents an alternative approach to biohydrogen production which may be better suited to mixed culture approaches (52, 58, 59) or versus cyanobacteria (46, 54). Creating mixed aerobic cultures is also interesting when considering sugar-releasing algae grown with a sugar consuming organism that produces both H_2 and NH_3 (11, 73).

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References

1. **Lineweaver H, Burk D, Horner CK.** 1932. The Temperature Characteristic of Respiration of *Azotobacter*. The Journal of General Physiology **15:**497-505.
2. **Shah VK, Ugalde RA, Imperial J, Brill WJ.** 1984. Molybdenum in Nitrogenase. Annu Rev Biochem **53:**231-257.
3. **Smith BE.** 2002. Structure. Nitrogenase Reveals its Inner Secrets. Science **297:**1654-1655.
4. **Barney BM, Lee HI, Dos Santos PC, Hoffman BM, Dean DR, Seefeldt LC.** 2006. Breaking the N_2 Triple Bond: insights into the Nitrogenase Mechanism. Dalton Trans**:**2277-2284.
5. **Burgess BK, Lowe DJ.** 1996. Mechanism of Molybdenum Nitrogenase. Chem Rev **96:**2983-3011.
6. **Barney BM, Plunkett MH, Natarajan V, Mus F, Knutson CM, Peters JW.** 2017. Transcriptional Analysis of an Ammonium-Excreting Strain of *Azotobacter vinelandii* Deregulated for Nitrogen Fixation. Appl Environ Microbiol **83**.
7. **Hamilton TL, Ludwig M, Dixon R, Boyd ES, Dos Santos PC, Setubal JC, Bryant DA, Dean DR, Peters JW.** 2011. Transcriptional Profiling of Nitrogen Fixation in *Azotobacter vinelandii*. J Bacteriol **193:**4477-4486.
8. **Setubal JC, dos Santos P, Goldman BS, Ertesvåg H, Espin G, Rubio LM, Valla S, Almeida NF, Balasubramanian D, Cromes L, Curatti L, Du Z, Godsy E, Goodner B, Hellner-Burris K, Hernandez JA, Houmiel K, Imperial J, Kennedy C, Larson TJ, Latreille P, Ligon LS, Lu J, Maerk M, Miller NM, Norton S, O'Carroll IP, Paulsen I, Raulfs EC, Roemer R, Rosser J, Segura D, Slater S, Stricklin SL, Studholme DJ, Sun J, Viana CJ, Wallin E, Wang B, Wheeler C, Zhu H, Dean DR, Dixon R, Wood D.** 2009. Genome Sequence of *Azotobacter vinelandii*, an Obligate Aerobe Specialized To Support Diverse Anaerobic Metabolic Processes. J Bacteriol **191:**4534-4545.

9. **Little R, Martinez-Argudo I, Dixon R.** 2006. Role of the Central Region of NifL in Conformational Switches that Regulate Nitrogen Fixation. Biochem Soc Trans **34:**162-164.
10. **Bali A, Blanco G, Hill S, Kennedy C.** 1992. Excretion of Ammonium by a *nifL* Mutant of *Azotobacter vinelandii* Fixing Nitrogen. Appl Environ Microbiol **58:**1711-1718.
11. **Barney BM, Eberhart LJ, Ohlert JM, Knutson CM, Plunkett MH.** 2015. Gene Deletions Resulting in Increased Nitrogen Release by *Azotobacter vinelandii*: Application of a Novel Nitrogen Biosensor. Appl Environ Microbiol **81:**4316-4328.
12. **Brewin B, Woodley P, Drummond M.** 1999. The Basis of Ammonium Release in *nifL* Mutants of *Azotobacter vinelandii*. J Bacteriol **181:**7356-7362.
13. **Ortiz-Marquez JC, Do Nascimento M, Curatti L.** 2014. Metabolic Engineering of Ammonium Release for Nitrogen-Fixing Multispecies Microbial Cell-Factories. Metab Eng **23:**154-164.
14. **Ortiz-Marquez JC, Do Nascimento M, Dublan MD, Curatti L.** 2012. Association with an Ammonium-Excreting Bacterium Allows Diazotrophic Culture of Oil-Rich Eukaryotic Microalgae. Appl Environ Microbiol **78:**2345-2352.
15. **Schreiber F, Littmann S, Lavik G, Escrig S, Meibom A, Kuypers MMM, Ackermann M.** 2016. Phenotypic Heterogeneity Driven by Nutrient Limitation Promotes Growth in Fluctuating Environments. Nat Microbiol **1:**7.
16. **Burk D, Horner CK, Lineweaver H.** 1932. Injury and Recovery of Respiration and Catalase Activity in *Azotobacter*. The Journal of Cellular and Comparative Physiology **1:**435-449.
17. **Newton JW, Wilson PW, Burris RH.** 1953. Direct Demonstration of Ammonia as an Intermediate in Nitrogen Fixation by *Azotobacter*. J Biol Chem **204:**445-451.
18. **Jensen HL.** 1954. The Azotobacteriaceae. Bacteriological Reviews **18:**195-214.
19. **Koch B, Evans HJ, Russell S.** 1967. Properties of the Nitrogenase System in Cell-Free Extracts of Bacteroids from Soybean Root Nodules. Proc Natl Acad Sci U S A **58:**1343-1350.

20. **Guth JH, Burris RH.** 1983. Inhibition of Nitrogenase-Catalyzed NH_3 Formation by H_2. Biochemistry **22:**5111-5122.
21. **Meletzus D, Rudnick P, Doetsch N, Green A, Kennedy C.** 1998. Characterization of the *glnK-amtB* Operon of *Azotobacter vinelandii*. J Bacteriol **180:**3260-3264.
22. **Martinez-Argudo I, Little R, Shearer N, Johnson P, Dixon R.** 2004. The NifL-NifA System: a Multidomain Transcriptional Regulatory Complex that Integrates Environmental Signals. J Bacteriol **186:**601-610.
23. **Ambrosio R, Ortiz-Marquez JCF, Curatti L.** 2017. Metabolic Engineering of a Diazotrophic Bacterium Improves Ammonium Release and Biofertilization of Plants and Microalgae. Metab Eng **40:**59-68.
24. **Apte SK, Chaurasia AK.** 2011. Improved Eco-Friendly Recombinant *Anabaena sp*. Strain PCC7120 with Enhanced Nitrogen Biofertilizer Potential. Appl Environ Microbiol **77:**395-399.
25. **Leaungvutiviroj C, Ruangphisarn P, Hansanimitkul P, Shinkawa H, Sasaki K.** 2010. Development of a New Biofertilizer with a High Capacity for N_2 Fixation, Phosphate and Potassium Solubilization and Auxin Production. Biosci Biotechnol Biochem **74:**1098-1101.
26. **Dos Santos PC.** 2011. Molecular Biology and Genetic Engineering in Nitrogen Fixation. Methods Mol Biol **766:**81-92.
27. **Eberhart LJ, Knutson CM, Barney BM.** 2016. A Methodology for Markerless Genetic Modifications in *Azotobacter vinelandii*. J Appl Microbiol **120:**1595-1604.
28. **Lenneman EM, Ohlert JM, Palani NP, Barney BM.** 2013. Fatty Alcohols for Wax Esters in *Marinobacter aquaeolei* VT8: Two Optional Routes in the Wax Biosynthesis Pathway. Appl Environ Microbiol **79:**7055-7062.
29. **Noar J, Loveless T, Navarro-Herrero JL, Olson JW, Bruno-Bárcena JM.** 2015. Aerobic Hydrogen Production via Nitrogenase in *Azotobacter vinelandii* CA6. Appl Environ Microbiol **81:**4507-4516.
30. **Noar JD, Bruno-Bárcena JM.** 2016. Protons and Pleomorphs: Aerobic Hydrogen Production in *Azotobacters*. World J Microbiol Biotechnol **32:**8.

31. **Barney BM, Igarashi RY, Dos Santos PC, Dean DR, Seefeldt LC.** 2004. Substrate Interaction at an Iron-Sulfur Face of the FeMo-Cofactor During Nitrogenase Catalysis. J Biol Chem **279:**53621-53624.

32. **Barney BM, Lukoyanov D, Igarashi RY, Laryukhin M, Yang TC, Dean DR, Hoffman BM, Seefeldt LC.** 2009. Trapping an Intermediate of Dinitrogen (N_2) Reduction on Nitrogenase. Biochemistry **48:**9094-9102.

33. **Hill S, He LH, Kennedy C.** 1999. Physiological Characterisation of an *Azotobacter vinelandii nifU*-Deletion Mutant and its Spontaneous Nif^+ Revertants that Over-Produce Cytochrome *bd*. FEMS Microbiol Lett **175:**185-191.

34. **Martin AE, Burgess BK, Iismaa SE, Smartt CT, Jacobson MR, Dean DR.** 1989. Construction and Characterization of an *Azotobacter vinelandii* Strain with Mutations in the Genes Encoding Flavodoxin and Ferredoxin I. J Bacteriol **171:**3162-3167.

35. **Strandberg GW, Wilson PW.** 1968. Formation of the Nitrogen-Fixing Enzyme System in *Azotobacter vinelandii*. Can J Microbiol **14:**25-31.

36. **Wong TY, Maier RJ.** 1985. H_2-Dependent Mixotrophic Growth of N_2-Fixing *Azotobacter vinelandii*. J Bacteriol **163:**528-533.

37. **Jain DK, Beyer D, Rennie RJ.** 1987. Dinitrogen Fixation (C_2H_2 Reduction) by Bacterial Strains at Various Temperatures. Plant and Soil **103:**233-237.

38. **Miller RW, Eady RR.** 1988. Molybdenum and Vanadium Nitrogenases of *Azotobacter chroococcum* - Low Temperature Favours N_2 Reduction by Vanadium Nitrogenase. Biochem J **256:**429-432.

39. **Walmsley J, Kennedy C.** 1991. Temperature-Dependent Regulation by Molybdenum and Vanadium of Expression of the Structural Genes Encoding Three Nitrogenases in *Azotobacter vinelandii*. Appl Environ Microbiol **57:**622-624.

40. **Dilworth MJ, Eady RR.** 1991. Hydrazine is a Product of Dinitrogen Reduction by the Vanadium-Nitrogenase from *Azotobacter chroococcum*. Biochem J **277:**465-468.

41. **Dilworth MJ, Eldridge ME, Eady RR.** 1993. The Molybdenum and Vanadium Nitrogenases of *Azotobacter chroococcum*: Effect of Elevated Temperature on N_2 Reduction. Biochem J **289:**395-400.
42. **Inomura K, Bragg J, Follows MJ.** 2017. A Quantitative Analysis of the Direct and Indirect Costs of Nitrogen Fixation: a Model Based on *Azotobacter vinelandii*. The ISME journal **11:**166-175.
43. **Finn RK.** 1954. Agitation-Aeration in the Laboratory and in Industry. Bacteriol Rev **18:**254-274.
44. **Wichard T, Bellenger JP, Morel FMM, Kraepiel AML.** 2009. Role of the Siderophore Azotobactin in the Bacterial Acquisition of Nitrogenase Metal Cofactors. Environ Sci Technol **43:**7218-7224.
45. **Rey FE, Heiniger EK, Harwood CS.** 2007. Redirection of Metabolism for Biological Hydrogen Production. Appl Environ Microbiol **73:**1665-1671.
46. **Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wünschiers, R., Lindblad P.** 2002. Hydrogenases and Hydrogen Metabolism of Cyanobacteria. Microbiol Mol Biol R **66:**1-20.
47. **Linkerhägner K, Oelze J.** 1995. Hydrogenase Does Not Confer Significant Benefits to *Azotobacter vinelandii* Growing Diazotrophically Under Conditions of Glucose Limitation. J Bacteriol **177:**6018-6020.
48. **Barnes EM, Jr., Zimniak P.** 1981. Transport of Ammonium and Methylammonium Ions by *Azotobacter vinelandii*. J Bacteriol **146:**512-516.
49. **Ludewig U, Neuhäuser B, Dynowski M.** 2007. Molecular Mechanisms of Ammonium Transport and Accumulation in Plants. FEBS letters **581:**2301-2308.
50. **Musa-Aziz R, Jiang LH, Chen LM, Behar KL, Boron WF.** 2009. Concentration-Dependent Effects on Intracellular and Surface pH of Exposing *Xenopus* oocytes to Solutions Containing NH3/NH4+. J Membrane Biol **228:**15-31.
51. **Winkler FK.** 2006. Amt/MEP/Rh Proteins Conduct Ammonia. Pflugers Arch **451:**701-707.

52. **Rittmann S, Herwig C.** 2012. A Comprehensive and Quantitative Review of Dark Fermentative Biohydrogen Production. Microb Cell Fact **11:**18.
53. **Bandyopadhyay A, Stockel J, Min HT, Sherman LA, Pakrasi HB.** 2010. High Rates of Photobiological H-2 Production by a Cyanobacterium Under Aerobic Conditions. Nat Commun **1:**7.
54. **Masukawa H, Inoue K, Sakurai H, Wolk CP, Hausinger RP.** 2010. Site-Directed Mutagenesis of the *Anabaena* sp. Strain PCC 7120 Nitrogenase Active Site To Increase Photobiological Hydrogen Production. Appl Environ Microbiol **76:**6741-6750.
55. **Weyman PD, Pratte B, Thiel T.** 2010. Hydrogen Production in Nitrogenase Mutants in *Anabaena variabilis*. FEMS Microbiol Lett **304:**55-61.
56. **Ghirardi ML, King PW, Posewitz MC, Maness PC, Fedorov A, Kim K, Cohen J, Schulten K, Seibert M.** 2005. Approaches to Developing Biological H-2-Photoproducing Organisms and Processes. Biochem Soc Trans **33:**70-72.
57. **Bothe H, Schmitz O, Yates MG, Newton WE.** 2010. Nitrogen Fixation and Hydrogen Metabolism in Cyanobacteria. Microbiol Mol Biol R **74:**529-551.
58. **Elsharnouby O, Hafez H, Nakhla G, El Naggar MH.** 2013. A Critical Literature Review on Biohydrogen Production by Pure Cultures. Int J Hydrogen Energ **38:**4945-4966.
59. **Sinha P, Pandey A.** 2011. An Evaluative Report and Challenges for Fermentative Biohydrogen Production. Int J Hydrogen Energ **36:**7460-7478.
60. **Radakovits R, Jinkerson RE, Darzins A, Posewitz MC.** 2010. Genetic Engineering of Algae for Enhanced Biofuel Production. Eukaryot Cell **9:**486-501.
61. **Shaw S, Lukoyanov D, Danyal K, Dean DR, Hoffman BM, Seefeldt LC.** 2014. Nitrite and Hydroxylamine as Nitrogenase Substrates: Mechanistic Implications for the Pathway of N_2 Reduction. Journal of the American Chemical Society **136:**12776-12783.

62. **Barahona E, Jiménez-Vicente E, Rubio LM.** 2016. Hydrogen Overproducing Nitrogenases Obtained by Random Mutagenesis and High-Throughput Screening. Sci Rep **6:**9.

63. **Dos Santos PC, Mayer SM, Barney BM, Seefeldt LC, Dean DR.** 2007. Alkyne Substrate Interaction Within the Nitrogenase MoFe Protein. Journal of Inorganic Biochemistry **101:**1642-1648.

64. **Barney BM, Laryukhin M, Igarashi RY, Lee HI, Dos Santos PC, Yang TC, Hoffman BM, Dean DR, Seefeldt LC.** 2005. Trapping a Hydrazine Reduction Intermediate on the Nitrogenase Active Site. Biochemistry **44:**8030-8037.

65. **Pham DN, Burgess BK.** 1993. Nitrogenase Reactivity: Effects of pH on Substrate Reduction and CO Inhibition. Biochemistry **32:**13725-13731.

66. **Fixen KR, Zheng YN, Harris DF, Shaw S, Yang ZY, Dean DR, Seefeldt LC, Harwood CS.** 2016. Light-Driven Carbon Dioxide Reduction to Methane by Nitrogenase in a Photosynthetic Bacterium. Proc Natl Acad Sci USA **113:**10163-10167.

67. **Schütz K, Happe T, Troshina O, Lindblad P, Leitão E, Oliveira P, Tamagnini P.** 2004. Cyanobacterial H_2 Production - a Comparative Analysis. Planta **218:**350-359.

68. **Curatti L, Brown CS, Ludden PW, Rubio LM.** 2005. Genes Required for Rapid Expression of Nitrogenase Activity in *Azotobacter vinelandii*. Proc Natl Acad Sci USA **102:**6291-6296.

69. **Klugkist J, Haaker H.** 1984. Inhibition of Nitrogenase Activity by Ammonium Chloride in *Azotobacter vinelandii*. J Bacteriol **157:**148-151.

70. **Gutierrez JC, Santero E, Tortolero M.** 1997. Ammonium Repression of the Nitrite-Nitrate (*nasAB*) Assimilatory Operon of *Azotobacter vinelandii* is Enhanced in Mutants Expressing the *nifO* Gene at High Levels. Mol Gen Genet **255:**172-179.

71. **Liang JH, Nielsen GM, Lies DP, Burris RH, Roberts GP, Ludden PW.** 1991. Mutations in the *draT* and *draG* Genes of *Rhodospirillum rubrum* Result in Loss of Regulation of Nitrogenase by Reversible ADP-Ribosylation. J Bacteriol **173:**6903-6909.

72. **Rubel ET, Raittz RT, Coimbra NA, Gehlen MA, Pedrosa FO.** 2016. ProClaT, a New Bioinformatics Tool for *In Silico* Protein Reclassification: Case Study of DraB, a Protein Coded from the *draTGB* Operon in *Azospirillum brasilense*. Bmc Bioinformatics **17:**455.

73. **Arriola MB, Velmurugan N, Zhang Y, Plunkett MH, Hondzo H, Barney BM.** 2017. Genome Sequences of *Chlorella sorokiniana* UTEX 1602 and *Micractinium conductrix* SAG 241.80: Implications to Maltose Excretion by a Green Alga. The Plant journal : for cell and molecular biology doi:10.1111/tpj.13789.